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THE EFFECT OF PITUITRIN ON FLUID DISTRIBUTION IN *BUFO REGULARIS* REUSS

BY R. F. EWER

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(*Received 4 July 1951*)

I. INTRODUCTION

While investigating the effects of posterior pituitary extracts on water balance in *Bufo regularis* a new physiological action of the extracts was noticed. The normal experimental procedure was to keep the animals in water and to measure the rates of water uptake and of urine output. If, however, the animals are injected with pituitrin and then kept dry, instead of being replaced in water, the lymph sacs of the thighs are found to become distended with fluid. At room temperature the effect is maximal about 2 hr. after injection, and passes off after about 4 hr. The magnitude of the effect is very variable, but is often so great that the skin at the bases of the thighs and ventral to the anus hangs down in a baggy fluid-filled pouch. Controls injected with saline show no such change. The results of some preliminary investigations of this effect are given below.

II. RESULTS

The species used was *Bufo regularis* Reuss, and all experiments were carried out at 24–26° C. The pituitary extract used was Parke Davis Pituitrin.

A quantitative estimate of the extent of fluid accumulation in the lymph spaces was first necessary. Eight pairs of toads, matched for size, sex and general condition, were selected and kept in water at 26° C. for 1 hr. to ensure that they were fully hydrated. They were then removed from the water, and one member of each pair was injected with pituitrin at a dosage of 3 i.u./100 g. body weight, while the other received a corresponding volume of 0·65 % saline. The animals were then kept for 2 hr. in closed containers over damp cotton-wool. At the end of this period the animals were killed, and the fluid in the lymph sacs of the thighs was pipetted off and weighed. The coelom is in communication with the lymph spaces, the body cavity was therefore opened and the fluid in it similarly removed and weighed. Table 1 shows the mean values of these weights for the eight pairs of animals, expressed as percentages of body weight. The excess percentage for each injected animal, as compared with its control, was also calculated, and the mean values are included in the table. The differences between the pituitrin-injected animals and the controls are significant: for lymph-sac fluid $P < 0{\cdot}01$ and for coelomic fluid P lies between 0·05 and 0·02. In all eight cases the pituitrin-injected animals contained more fluid in their lymph sacs than did their controls, while seven out of eight contained more fluid in the coelom also.

It was previously noted (Ewer, 1950) that in toads which are kept in water after pituitrin injection the excess water load which is taken up is largely retained in the lymph spaces and results in little general hydration of the tissues. The present observations show that this accumulation of fluid in the lymph sacs is not conditional on the presence of excess fluid in the animal, and it at once became of interest to find the source of the extra lymphatic fluid when free water is not available to the animal. A series of experiments to investigate this point was carried out. Pairs of matched toads were treated as in the experiment just described. Two hours after injection the animals were examined, and in all cases the lymph sacs of the pituitrin-injected animals were distended with fluid, while those of the saline-injected controls were not. The animals were then pithed and various tissues removed as rapidly as possible for water-content determinations. The tissues used were the skin from the 'waist' down, the two gastrocnemius muscles, the gut and the liver. The procedure was as described previously (Ewer, 1950). The values found for the water contents of the tissues of the pituitrin-injected animals and of the saline-injected controls are given in Table 2. In no case is the difference significant. This result was unexpected, but might be interpreted as indicating that the experimental errors in drying and weighing the tissues were so great as to mask any effect of the injection. The excess percentages of fluid found in the lymph sacs and body cavities of pituitrin-injected animals are not very large, amounting in all to only 1·5 % of the body weight (see Table 1). It must, however, be borne in mind that since accumulation of fluid has been found in both the body cavity and the lymph sacs of the thighs, it probably takes place in all lymph spaces, and indeed can be seen to occur in the dorsal lymph space: the total accumulation of fluid is therefore greater than the 1·5 % actually measured.

Table 1. *Weights of fluid contained in lymph sacs and in body cavity in pituitrin-injected toads as compared with saline-injected controls*

(The values are expressed as percentages of total body weight, and each figure is the mean of determinations made on eight animals.)

	Lymph sac.	Body cavity
Pituitrin	1·06	1·13
Saline	0·22	0·47
Difference	0·84 ± 0·12	0·66 ± 0·23

Table 2. *Percentage water contents of the tissues of pituitrin-injected and of control saline-injected toads*

(Each figure is the mean of eight determinations.)

	Skin	Muscles	Gut	Liver
Pituitrin	74·81 ± 0·89	80·25 ± 0·78	80·35 ± 0·72	76·48 ± 0·96
Saline	75·33 ± 0·50	79·38 ± 0·61	81·35 ± 0·56	75·20 ± 0·61

During the course of the experiments just described, it was noticed that while the bladders of the control animals were frequently full of fluid those of the pituitrin-injected toads were always empty. This suggested that the pituitrin injection was causing resorption of fluid from the bladder, and that this was the principal source of the fluid accumulated in the lymph sacs. A further series of experiments was therefore carried out to test this possibility. From previous work it was known that when *B. regularis* is kept in water urine flow keeps pace with water uptake, so that in a period of an hour the weight of urine produced is approximately equal to the weight of water absorbed through the skin. After a preliminary period in water to ensure a state of balance the bladder can be emptied by means of a cannula, the cloaca ligatured, and the animal replaced in water for a further period of 1 hr. At the end of this period the increase in weight is then approximately equal to the weight of urine in the bladder. This weight increase is referred to as the presumed bladder content (p.b.c.). As a check on the validity of this estimate of bladder content six animals were treated as described, the ligatures were immediately removed and the urine taken from the bladder and weighed. The mean value for the actual bladder content was found to be 103 % p.b.c.; the latter therefore gives a reasonably close approximation to the actual bladder content. Experiments were then carried out as follows. After a preliminary period to ensure hydration the bladder of the animal was emptied, the cloaca ligatured and the animal weighed and replaced in water for 1 hr. At the end of this period the p.b.c. was determined by weighing again, and the animal was injected with pituitrin at a dosage of 3 i.u./100 g. The toad was then placed in a closed vessel over damp cotton-wool and left for 2 hr., at the end of which time it was killed and the urine in the bladder removed and weighed. The amount of urine in the bladder was then expressed as a percentage of the p.b.c. The mean value found for seven pituitrin-injected animals was 6·7 %, the highest individual value being 29·5 % Five control saline-injected animals all had more urine in their bladders than the p.b.c.; the mean value for the bladder content at the end of the experiment in these controls was 150 % p.b.c., showing that there had been further secretion of urine during the 2 hr. period following injection. These experiments show that after pituitrin injection there is resorption of fluid from the bladder, whereas in the saline-injected controls this does not occur, provided the animals are kept in a moist atmosphere.

It is necessary for the animals to be kept in a moist atmosphere because in dry conditions resorption from the bladder may take place without pituitrin injection. There were indications that this might be so during the course of the observations just described; the point was therefore investigated in the following set of experiments. As before, the animals were kept in water for a preliminary period; the bladders were emptied, the cloacas ligatured and the animals weighed. They were then replaced in water for an hour and the p.b.c. determined as before by a second weighing. The animals were then placed in wire-covered dishes in a slight draught from an electric fan. In these conditions they lost water at the rate of 1–3 % per hour. After periods of 1, 2 or 4 hr. the ligatures were removed and the urine in the bladders collected and weighed. Table 3 shows the results. During the first 2 hr. in dry conditions there is a diminution in the amount of urine in the bladder, but by the end of 4 hr. the bladder content has started to rise again. These results indicate that resorption of fluid from the bladder may take place in normal uninjected animals in natural conditions. Steen (1929) records a similar resorption of urine in an anuran of unstated species when the animal was subjected to desiccation.

Experiments were undertaken to determine the relative effects of pitressin and pitocin on fluid accumulation in the lymph sacs and resorption from the bladder. It has been found that both pitressin and pitocin cause both these responses in *B. regularis*, but there are indications that there may be seasonal alterations in sensitivity: investigations on this point are still in progress.

Table 3. *Weight of urine remaining in the bladder at different times after the animals have been put in dry conditions*

(The figures are the weight of urine expressed as a percentage of the p.b.c.; each represents a single determination made on a single toad.)

0 hr.	1 hr.	2 hr.	4 hr.
104	83	65	143
115	47	28	95
125	34	52	51
85	84	18	74
100	77	67	56
88	—	—	58
Mean 103	65	46	80

III. CONCLUSION

The well-known effects of posterior pituitary extracts in increasing water uptake and reducing urine flow of Anura are of such a character as to be of biological significance in relation to the water economy of the animals. The matter is fully discussed by Jørgensen (1950). The two components of the new effect described above may similarly be expected to assist in water conservation. While the animal is in water copious dilute urine is secreted. If dry conditions are then encountered the fluid in the bladder need not necessarily be 'wasted' by being voided to the exterior: instead it may be resorbed, while at the same time fluid distribution within the animal is so altered as to keep the 'reserve' in the lymph sacs, leaving the water content of the tissues relatively unaffected.

In a previous paper (Ewer, 1951) the antidiuretic effects of posterior pituitary extracts on *B. regularis* are discussed. This antidiuretic effect consists of a decrease in the urine flow from the cannulated cloaca. Pituitrin has now been found to cause resorption of fluid from the bladder. It therefore appears possible that the observed diminution of urine flow after injection of pituitary extracts may be partly or wholly the result of such resorption from the bladder. This is, however, unlikely, since in the experiments in question the arrangement of the cannula in the cloaca is such that the urine does not usually remain in the bladder but flows directly down the cannula into the collecting balloon (see Ewer, 1950). Nevertheless, further experiments, distinguishing between a genuine antidiuresis with reduced urine secretion by the kidney and a diminished urine flow as a result of resorption in the bladder, are necessary before this point can be finally decided.

IV. SUMMARY

A new response to injection of posterior pituitary extract in *Bufo regularis* Reuss is described. If the animals are kept out of water after injection there is a significant increase in the fluid held in the lymph spaces, and fluid is resorbed from the bladder.

The accumulation of lymph is not accompanied by any significant decrease in the water content of the tissues.

The possible biological significance of the response is indicated.

This work was carried out during the tenure of a Research Grant from the South African Council for Scientific and Industrial Research.

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RETINOMOTOR AND DIURNAL RHYTHM IN CRUSTACEANS

By H. E. HENKES
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(*Received* 30 *July* 1951)

(With Plates 6–8 and Five Text-figures)

INTRODUCTION

It is well known that a periodicity in various vital functions is still maintained after the elimination of all periodic environmental factors. Formerly it was believed that these factors were essential to the preservation of such periodicity, and a preponderant role was ascribed to the light perceiving organ. Kiesel (1894), however, was the first to notice a persistent periodicity of luminosity in the compound eye (observable after a short illumination by means of an ophthalmoscope): this occurred even when the animal (*Plusia gamma*) was kept permanently placed in the dark. The change took place at sunset and was reversed at daybreak. The true significance of this phenomenon was realized only at a later date.

Diurnal rhythms are described not only in relation to vision, but also in relation to other physiological functions, e.g. the periodic alterations in the expansion of dermal chromatophores, as described in various forms of life by different investigators (Gamble & Keeble, 1900, in *Hippolyte varians*; Young, 1935, in *Lampetra planeri;* Hogben & Slome, 1931, in *Xenopus laevis*). However, some of the most interesting phenomena of this kind are the periodic changes in the pigment systems of the compound eye, which continue even when the animal is kept permanently in the dark. (For corresponding phenomena in the vertebrate eye see Welsh & Osborn, 1937; Arey & Mundt, 1941).

Demoll (1911) confirmed the observation of Kiesel. More recently, further investigations have been carried out by Welsh (1930*a*, 1932, 1936), who observed a diurnal rhythm in the distal pigment cells in *Macrobrachium* and *Anchistioides* (see Text-fig. 3) after elimination of all interfering factors, including light. Bennitt (1932*a*) noticed a similar rhythm in the proximal pigment cells of *Canbarus*. From these observations it is evident that more than one pigment system is involved in the diurnal rhythm.

The crustacean eye usually contains three pigment systems (see Text-fig. 1), although in some species only two are known. These are:

(*a*) The distal, so-called 'iris' pigment, which in dark adaptation envelops the crystal cone of each ommatidium.

(*b*) The proximal, so-called 'retina' pigment, lying in dark adaptation below the membrana fenestra.

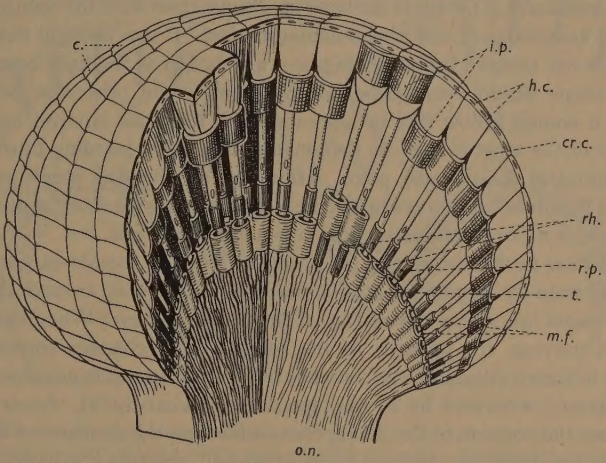
(*c*) The reflecting pigment or 'tapetum' consisting of an amorphous guanine mass. In dark adaptation this pigment is located above, or distal to, the membrana fenestra. The tapetum is responsible for the appearance of luminosity in the eye when the animal is placed in darkness.

During light-adaptation, the following changes take place in the three pigment systems:

(*a*) The iris pigment migrates in a proximal direction between the ommatidia.

(*b*) The retinal pigment migrates distally through the gaps in the membrana fenestra.

(*c*) The tapetum is retracted through the meshes of the membrana fenestra to give the retinal pigment an opportunity of enveloping the rhabdomes completely.



Text-fig. 1. Three-dimensional diagram of the compound eye in light-adapted state; only two ommatidia in the right half are shown in dark-adaptation. *c.* cornea; *h.c.* hypodermis cell; *cr.c.* crystal cone, distal part of the ommatidia; *i.p.* iris pigment; *r.p.* retinal pigment; *t.* tapetum; *o.n.* optic nerve fibres; *rh.* rhabdome, proximal part of the ommatidia.

Fig. 1 illustrates this whole process in a three-dimensional diagram. On the left side of the figure the pigment systems are shown in light-adaptation, whereas on the right side two ommatidia are shown in a state of dark-adaptation. (Compare with Pl. 7, fig. 1, *a* and *b*; sections of the eye of *L. squilla* in light and dark-adaptation respectively.)

When the diurnal rhythm is studied, it must not be forgotten that other important factors besides light play a role in the retinomotor process. Congdon (1907) demonstrated the influence of temperature on the position of the retinal pigment in *Palaemonetes* and *Cambarus*; Bennitt (1924) found that in the state of anaesthesia and 'post-mortem' the pigment systems of most Crustaceans assume the position of light-adaptation. Again, Bennitt, co-operating with Dickson Merrick (1932*b*), demonstrated the influence of lack of oxygen upon the position of the retinal pigment in *Cambarus*. The same state of light-adaptation can be demonstrated immediately before death and in overcrowded aquaria.

From the above it is clear that in the study of diurnal rhythm it is absolutely essential to pay attention not only to absolute darkness during observations, but also to constant temperature and sufficient oxygenation of the seawater. [For general aspects of retinomotor phenomena, the reader is referred to the excellent monographs of Exner (1891), of Hess (1912) and to a more recent one by von Studnitz (1940).]

Retinomotor changes can be studied by histological methods. In addition, a number of related phenomena, which can be studied *in vivo*, also depend on changes in the position of the pigment. These are:

A. *The luminosity of the eye in darkness* on illumination with the ophthalmoscope. Leydig had knowledge of this phenomenon in 1864. It was thought that when the tapetum was not covered by retinal pigment a reflexion of the light beam from the ophthalmoscope would occur, whereas this light would otherwise be absorbed. From this it should follow that in cases in which a diurnal rhythm in the retinal pigment or in the tapetum can be demonstrated, a corresponding diurnal rhythm in the luminosity should take place. However, the present investigations have shown that this does not always hold true, and therefore we must assume that this phenomenon is more complicated than is generally believed.

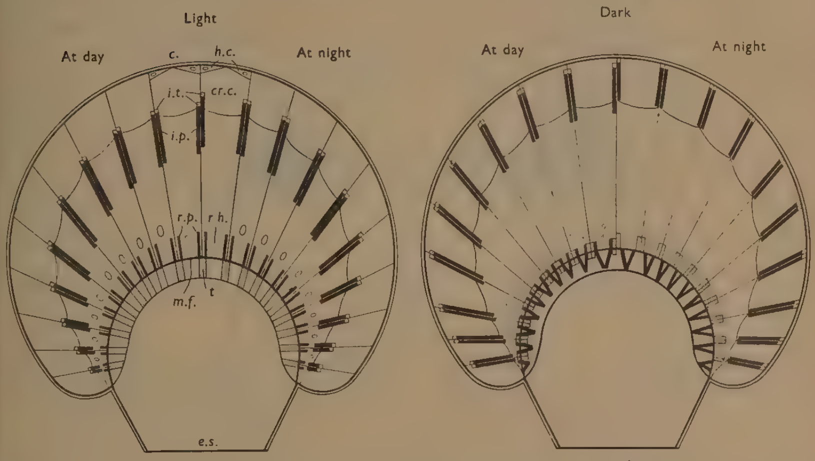
B. *Variations in the diameter of the pseudo-pupil.* The pseudo-pupil—described for the first time by Leydig (1855)—appears as a dark spot around the point at which the visual line meets the surface of the compound eye. When the eye of the observer is moving, the position of the pseudo-pupil changes correspondingly. According to Exner (1891), the appearance of a pseudo-pupil is possible only when the iris pigment is covered by an iris tapetum (see Text-fig. 2). According to the general view, the position of the iris tapetum influences the diameter of the pseudo-pupil. In light-adaptation the pseudo-pupil is small, whereas it is large in the dark-adapted eye. An oval or rectangular form of the pseudo-pupil can be explained either by differences in the position of the iris tapetum and iris pigment around the affected (round or square) ommatidia, or by an astigmatism of the cornea.

C. *Variations in the transillumination picture of the eye* (see Text-fig. 4). This investigation can be carried out only in transparent eyes such as those of *Leander*, which allow the light of a microscope lamp to pass through. The transparent margin around the dark central disk varies in breadth with the position of the iris pigment and the cornea of those ommatidia which lie in the horizontal plane perpendicular to the direction of observation (see Text-figs. 2 and 4). Welsh (1930*b*) made such measurements in the eyes of *Leander* and of *Anchistioides* in the course of his investigations on the diurnal rhythm (1936).

When studying the problem of the diurnal rhythm, I found it of great importance to compare the results obtained by studying the histological sections with those derived from the experiments *in vivo*, as described below.

The following crustaceans were used in the present investigations. The systematic diagnoses were made by Dott. Bacci in Naples. The number of bulbi of each species used is given below:

(1) *Leander squilla*	90 bulbi
(2) *Leander elegans*	70 ,,
(3) *Portunus arcuatus*	34 ,,
(4) *Portunus depurator*	14 ,,
(5) *Portunus corrugatus*	18 ,,
(6) *Pachygrapsus marmoratus*	20 ,,
(7) *Carcinus maenas*	40 ,,



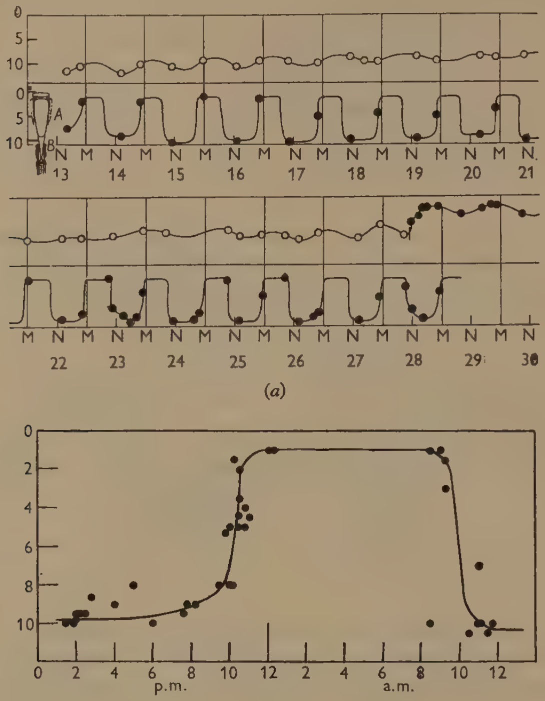
(*a*) (*b*)

Text-fig. 2. Showing the influence of the diurnal rhythm on the position of the pigment systems. This influence is most marked during dark-adaptation, see (*b*). (*a*) shows the pigment systems in light adaptation during the day and during the night respectively; (*b*) shows the migration of the pigment in the dark-adapted eye under the influence of the diurnal rhythm in daytime and at night. *c.* cornea; *h.c.* hypodermis cell; *cr.c.* crystal cone; *i.t.* iris tapetum; *i.p.* iris pigment; *rh.* rhabdome; *r.p.* retinal pigment; *m.f.* membrana fenestra; *t.* tapetum; *e.s.* eye stalk.

The investigations consisted in the examination of the dark-adapted eye:

(*a*) *In vivo.* (1) Luminosity of the eye on exposure to a light flash from the ophthalmoscope; (2) measurement of the diameter of the pseudo-pupil by means of an ocular-micrometer; (3) measurement of the dark disk and the breadth of the transparent zone by momentary trans-illumination of the eye of *Leander*. Although it is theoretically possible for a migration of pigment to occur as a result of a short exposure to light, this possibility may be disregarded since the migration process takes place too slowly (Welsh, 1930*b*). In the *Leander* eye full light-adaptation is not reached in under 50–60 min.

(*b*) *In histological section.* We measured the position of the distal 'iris' pigment, the proximal retina pigment and, if present, the reflecting pigment (tapetum) in relation to the cornea and the membrana fenestra. The histological technique used was a simple one. The animals were killed and simultaneously fixed by immersion in water at 80–90° C. The optic stalks were cut as close to the head as possible.



(a)

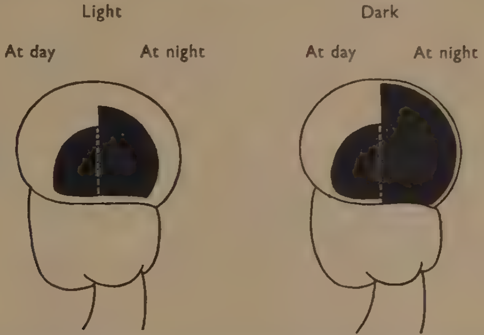
(b)

Text-fig. 3. (*a*) Records of the movements of the distal pigment cells of four animals kept in constant illumination (open circles), except for last 2 days, and of four animals kept in constant darkness (solid circles). Experiment started 13 June and ended 30 June. Noon and midnight are indicated by the letters *N* and *M*. In the sketch of an ommatidium *A* is the extreme peripheral or night position of the distant pigment cells, *B* the inner or day position, in constant darkness. The points as plotted are averages of the measurements of the distance from the cornea to the outer boundary of pigment. Each unit, 10 μ. Time of sunset during course of the experiment about 7.35 p.m. (*b*) The measurements made on the eyes of animals kept in constant darkness and shown plotted over a period of 15 days are here shown in one 24 hr. period. Such a plot makes it possible to construct a curve representing the daily movements of the distal pigment cells. One of the co-ordinates shows the hours of the day beginning at noon, the other the distance of the distal pigment cells from the outer boundary of the cornea, each unit being equal to 10 μ. (From Welsh, 1936.)

The eyes were placed successively in alcohol 96 %, alcohol 70 %, diaphanol (to soften the hard chitinous covering of the eyeball), alcohol 70 %, dioxan, dioxan-paraffin and paraffin.

The investigations were carried out on the unstained preparations. Two sections of each eye, including the optic nerve, were made. The two ommatidia most centrally situated were used for measurement. The presence of a tapetum was determined by means of dark-ground illumination. The iris and retinal pigment do not reflect, whereas the tapetum is visible as a brilliant white structure (see Pl. 6, fig. 1 *b*.)

The investigation of the diurnal rhythm was carried out under conditions of constant temperature (with a maximal variation between day and night of 0·3°), constant darkness and in well-ventilated aquaria. Specimens were taken before and after sunset.



Text-fig. 4. Eye of *Anchistioides antiguensis* showing the position of the distal iris pigment cells during constant illumination (left figure) and during constant darkness (right figure), during the day and viewed at night. (From Welsh, 1930.)

At the same time investigations into the retinomotor changes in general were carried out under conditions of (*a*) sunlight; (*b*) diffuse daylight; (*c*) post-mortem (after natural death); (*d*) darkness during the hours of daylight ('day-darkness'); (*e*) darkness during the hours of night ('night-darkness'). As far as possible, the eye was examined with the ophthalmoscope; the pseudo-pupil was measured and investigated by trans-illumination before death and the histological examination was performed thereafter as soon as possible.

There was no significant difference in the position of the pigment of animals killed (*a*) after 1 hr. exposure to sunlight, (*b*) after some hours in diffuse daylight, and (*c*) after natural death.

We are thus able to confirm Bennitt's (1932 *b*) findings that, irrespective of whether the animal has died naturally in sunlight or in darkness, the final position of the pigment is as found in the light-adapted state. The same holds true in all the Crustaceans investigated; and not only for the iris pigment, but also for the retinal pigment and the tapetum, if this is present. The influence of oxygen-lack to these findings will be dealt with later.

Text-fig. 2 *a* and *b* summarize in a diagram the migration of the three pigment systems in light- and dark-adaptation. The influence of the diurnal rhythm on the position of the pigment in the light- and dark-adapted eye is now discussed.

In adaptation to *normal illumination during the day* we find—as illustrated in Text-fig. 2*a* left half—the iris pigment (*i.p.*) and the tapetum (*t.*) situated in the extreme proximal position, whilst the retinal pigment (*r.p.*) is in the extreme distal position. The iris tapetum being in actual contact with the iris pigment, closely follows the movement of the latter in spite of an anatomical connexion between iris tapetum and tapetum (see Pl. 7). While the crystal cones of the ommatidia are deprived of iris pigment, the rhabdomes, on the other hand, are almost completely enveloped by a layer of retinal pigment.

During *illumination at night* ('light at night') (Text-fig. 2*a*, right half) the iris pigment lies more distally, indicating an initial change towards the dark-adapted position (Text-fig. 2*b*), although the positions of the retinal pigment and tapetum are unchanged. The effect of light changes upon the positions of retinal pigment and tapetum is much greater than the effect of the diurnal rhythm during constant illumination. The iris pigment, on the other hand, is influenced more by the diurnal rhythm.

When the animal is adapted to *dark during the night* ('night-darkness' position), the changes in position of the three pigment systems become evident. The iris pigment and tapetum migrate to the distal part, whilst the retinal pigment moves proximally (see Text-fig. 2*b*, right half).

As the rhabdomes are no longer covered by the retinal pigment, and as the latter is replaced completely by the light-reflecting tapetum, we observe a red glow of the pseudo-pupil. If the animal is kept *in the dark during* the hours of *daytime* (pigment in 'day-darkness' position; Text-fig. 2*b*, left half) these changes are not so marked; under the influence of the diurnal rhythm the iris pigment and tapetum move less distally and the retinal pigment moves less proximally. The glow of the pseudo-pupil now disappears because the reflecting tapetum is covered once again by the retinal pigment, and its glow will reappear only after sunset, when the influence of the diurnal rhythm is eliminated and final dark-adaptation has been established once again.

In some of the Crustaceans examined, it was obvious that the pigment changes, as described in Text-fig. 2, were incomplete. In *Leander*, for example, the tapetum is situated to a large extent above the membrana fenestra, even in extreme light-adaptation, i.e. in sunlight, whilst in *Leander* in dark-adaptation the retinal pigment is not found wholly under the membrana fenestra. Nevertheless, in general the changes of the pigment follow the scheme as outlined in Text-fig. 2*a* and *b*.

EXPERIMENTS

We tried to answer the following questions:

(A) Does a diurnal rhythm occur in the compound eyes of all species of Crustaceans?

(B) Are the same elements concerned in this periodicity of pigment migration in different species of Crustaceans when all light has been excluded, and does such a periodicity correspond with sunset and sunrise?

(C) Does a correlation exist between the position of the migrating pigment, the size of the pseudo-pupil and the glow of the eye? If so, can the existence of a diurnal rhythm be deduced by measuring the variations in size of the pseudo-pupil?

(D) What is the anatomical and physiological basis of the process of the diurnal rhythm?

Questions A and B

Although I was able to demonstrate a periodicity in each of the species of Crustaceans investigated, the changes may not in all cases take place in the same pigment system (as found in the investigations of Welsh, 1935, and Bennitt, 1932*a*).

Under optimal conditions and during a period of 14 days we found:

(1) In *Leander squilla*

(*a*) No marked difference between the position of the iris pigment in 'night-darkness' and 'day-darkness' as determined by means of trans-illumination *in vivo* and measured in the histological preparations. In various sections of eyes fixed under 'day-darkness' the iris pigment had shifted over a distance of about 10 μ, compared with the eyes of animals killed under 'night-darkness'. (Compare with this: Text-fig. 3, which shows the diurnal rhythm of the iris pigment in *Anchistioides* (Welsh, 1936), where migration took place over a distance of about 90 μ.)

(*b*) We could not discover a periodicity of any importance in the migration of retinal pigment in histological section, whereas

(*c*) A marked periodicity was demonstrated in the position of the tapetum; when fixed in the position of 'day-darkness' it was found that the distal border had moved about 15 μ proximally, measured from the membrana fenestra; simultaneously a diffuse distribution of the tapetum under the membrana fenestra was seen. (Compare Pl. 7*b* with *c*; sections of the eye of *Leander squilla* in 'night-darkness' and 'day-darkness' respectively.)

(2) In *Leander elegans*

The changes in pigment positions are in principle the same as in *L. squilla*. The investigation was carried out over a period of 9 days. A difference of 10 μ was measured between the distal border of the tapetum in 'night-darkness' and 'day-darkness' positions (see Pl. 8).

(3) In *Portunus arcuatus*

(*a*) I could not observe a movement in the iris pigment of an amount sufficient to establish with certainty the occurrence of a diurnal rhythm.

(*b*) In some preparations a detectable movement in the retinal pigment through the membrana fenestra in the distal direction could be demonstrated in 'day-darkness', whilst in other preparations under exactly the same conditions a significant migration of about 45 μ took place.

(*c*) An absence of the tapetum has been noted.

(4) In *Portunus depurator*

(*a*) A marked diurnal rhythm occurs in the position of the iris pigment; the pigment migrates from the 'night-darkness' to the 'day-darkness' position over a distance of 50 μ, measured from the cornea.

(*b*) The retinal pigment shows also a significant diurnal rhythm. When the 'day-darkness' position is reached we found a migration through the membrane likewise over a distance of 50 μ.

(*c*) A tapetum could not be demonstrated.

(5) In *Portunus corrugatus* we found

(*a*) A marked diurnal rhythm in the iris pigment (as in *P. depurator*) but only an average of 15 μ, whereas

(*b*) In *P. arcuatus* and *P. depurator* the diurnal rhythm resulted in a migration of the retinal pigment between 'night darkness' and 'day-darkness' positions over a distance of about 80 μ.

(*c*) Contrary to the finding of Exner (1891), I was able to demonstrate the presence of a tapetum; there was no significant alteration between 'night-darkness' and 'day-darkness' positions.

(6) In *Pachygrapsus marmoratus*

(*a*, *b*) No clear diurnal rhythm in the position of the iris-pigment nor in that of the retinal pigment was demonstrable, whereas

(*c*) In the tapetum, by contrast, a definite diurnal rhythm could be shown, with a migration of the pigment of about 60 μ in a proximal direction, when the state of 'day-darkness' is reached.

(7) In *Carinus maenas*

We could not detect:

(*a*) A diurnal rhythm in the iris pigment, although

(*b*) In the retinal pigment a definite diurnal rhythm could be shown. This pigment, which in the 'night-darkness' state is totally under (proximal to) the membrana fenestra, moves distally after sunrise (in the state of 'day-darkness') passing between the ommatidia and joins the iris pigment. A precise measurement is not possible, but the migration is roughly 130 μ (see Pl. 6, fig. 2*a*, *b*).

Question C

According to Welsh (1936) the transparent zone, demonstrable by trans-illumination of the transparent eye, narrows and broadens in accordance with the movement of the iris pigment, and its breadth depends on the distance between the distal border of the iris pigment and the cornea (see Fig. 4).

Besides this correlation between the breadth of the transparent zone and the position of the iris pigment which I was able to show, I also found a correlation between the position of the iris pigment measured in histological section and the diameter of the pseudo-pupil measured *in vivo*.

In the third place I demonstrated the relationship *in vivo* between the size of the pseudo-pupil and the breadth of the transparent border zone of the eye of *Leander*; measurements were made during a momentary trans-illumination and are summarized in Table 1. The data of both series of observations (*a*) and (*b*) show, according to Fischer (1946), a correlation in the form of a linear regression.

Table 1

(*a*)	1130	1575	1600	1100	1405	1415	880	1150	1450
(*b*)	160	370	310	140	250	305	130	140	200

(*a*) Diameter (in μ) of the dark centre of the compound eye during trans-illumination. (*b*) Diameter in μ of the pseudo-pupil. Both measured in nine animals.

(On account of the shape of the ommatidia of *Leander* (four-sided pyramids, with their bases against the cornea) the pseudo-pupil presents a square aspect. When measurements are made in an astigmatic part of the cornea, the pseudo-pupil becomes rectangular. The average length of the sides of the rectangle is taken to represent the diameter of the pseudo-pupil.)

Our investigation made it clear that this relationship between the size of the pseudo-pupil and the position of the iris pigment holds not only for the translucent eye, but also for the non-translucent eye of some of the other species of Crustaceans (*Portunus*, *Pachygrapsus* and *Carcinus*). In the latter the failure of the trans-illumination is due to the pigment in the hypodermis (Text-fig. 2). This pigment has absolutely no connexion with the pigment systems concerned with the retinomotor function.

Although for several weeks we measured the pseudo-pupil in *C. maenas in vivo* under diurnal rhythm conditions, no significant differences were noticed between the night and day recordings. This is in complete agreement with our findings on the histological preparations of the control animals, where there was no significant migration of the iris pigment.

Where the size of the pseudo-pupil and the breadth of the transparent zone of the eye both depend on the position of the iris pigment—as we have shown before—trans-illumination and measurement of the pseudo-pupil are valuable means of indicating *in vivo* the presence or absence of a diurnal rhythm in the iris pigment system.

We mentioned on p. 180, that periodic changes in the positions of the retinal pigment or of the tapetum can produce a diurnal rhythm in the luminosity of the eye. This latter change can be observed in the dark during the day and at night by means of an ophthalmoscope. We could demonstrate such a rhythm in the luminosity of the eye only in *Portunus arcuatus*, *P. depurator* and *Pachygrapsus marmoratus*.

Exner's (1891) explanation of the luminosity of the compound eye depends upon the light-reflecting tapetum being covered by the retinal pigment. Accepting this hypothesis, the diurnal rhythm in the luminosity of the eye can be explained by assuming such a rhythm in either or both tapetum and retinal pigment. However, neither *Portunus arcuatus* nor *P. depurator* possess a tapetum, though both display a diurnal rhythm in their retinal pigment. On the other hand, *P. corrugatus* possesses a tapetum, and exhibits a rhythm in the migration of the retinal pigment over a distance of 80 μ, thus providing ideal circumstances for the appearance of a diurnal rhythm in luminosity; but we never observed in this species any trace of luminosity of the pseudo-pupil. On the other hand, in *Leander* we observed an intense and invariable red glow of the pseudo-pupil, both in 'night-darkness' and 'day-darkness'. *Leander* shows the same ocular structure and the same diurnal rhythm in the tapetum as *Pachygrapsus marmoratus*, but *Pachygrapsus* shows a marked diurnal rhythm in luminosity which *Leander* does not.

From these facts, it will be clear that the explanation of this phenomenon is more complicated than was formerly believed. One can but state that the periodic changes in the luminosity of the pseudo-pupil *in vivo* are not conclusive evidence for the existence of a diurnal rhythm in tapetum or retinal pigment.

Question D

The cause of the diurnal rhythm has been the subject of speculation by many writers. For example, Hogben & Slome (1931) have ascribed it to hormonal influences, whilst Welsh (1936) has stressed the 'eyestalk hormone' of Kleinholz (1934) as an important aetiological factor in the rhythm of the distal pigment system. Bennitt (1932) ascribed the periodic changes to nervous factors, acting through the influence of periodic alterations of oxygen tension upon the cells of the different pigment systems concerned. Periodic changes in the metabolism have been suggested by Menke (1911). According to Bennitt (1932*a–c*) the aetiological factors could exert their actions via nervous or vascular pathways. From the diversity of opinions expressed by various experimental workers, the reader will appreciate that up to the present no definite conclusion has been reached as to the aetiology of this interesting problem. However, we must concede that changes in the metabolism of the pigment systems concerned may be a factor of importance.

Not much work has been done on the metabolism of Crustaceans and only a few facts are available (Henze, 1910; Cohnheim, 1911–12).

In *Carcinus* Cohnheim (1911–12) found, *inter alia*, an oxygen consumption of 7·4–10·1 mg./100 g./hr., and a carbon dioxide production of 15–27 mg./100 g./hr., with a respiratory quotient of 1·34–1·18.

Continuous estimations of metabolic rate in Crustaceans, a necessary requirement for our purpose, have not yet been made.

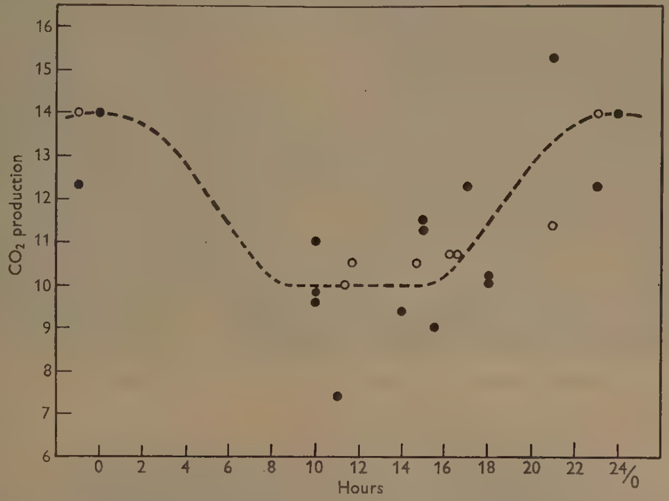
Since *C. maenas* shows a marked diurnal rhythm in the retinal pigment system (p. 186), and one specimen is large enough to allow a reliable determination of the metabolic rate, I chose this animal for the experiment.

The continuous metabolism estimations were performed by means of the diaferometer of Noyons (1937). Two series of experiments were done under different conditions:

(1) The animal in diffuse daylight (during the hours of daytime) and in darkness (during the hours of night).

(2) The animal permanently in the dark under conditions suitable for the study of the diurnal rhythm.

We thought it of interest to see if it was possible to demonstrate the presence of a diurnal rhythm in the production of carbon dioxide, when such measurements were made over a sufficient period of time. The results are shown in Text-fig. 5. From our data it is evident that during the 24 hr. period, even in constant darkness, the production of carbon dioxide is greater during the night than during the hours of daylight, thus indicating a diurnal rhythm in metabolism.



Text-fig. 5. Curve showing the continuous determination of the metabolism in *Carcinus maenas*. Measurements are shown in one 24 hr. period. From the data obtained a curve can be constructed representing the daily variations in the production of carbon dioxide. On the abscissa time is plotted in hours from 0 to 24. The relative carbon dioxide production is plotted on the ordinate. First series (open circles): the animal is kept in dimmed daylight during the day and in darkness during the night; second series (filled circles): animal in continuous darkness. Note that there is no marked difference between these two series.

The sea-water could not be changed in the course of the experiment; consequently the animals showed signs of intoxication, probably due to the presence of their own excreta. For this reason it was impossible to continue with the investigations beyond a period of 3 days.

The series of experiments were made: First series (open circles): the animal was kept in diffuse daylight during the day; in darkness at night. Second series (solid circles): the animal was kept in complete darkness.

Before we started with the second series, the animal was adapted to constant darkness for a period of several days. In both series a constant temperature and adequate oxygenation of the sea-water were maintained.

We have demonstrated—at least in *Carcinus*—a diurnal metabolic rhythm in terms of a diurnal rhythm in the production of carbon dioxide, which is about

30 % smaller by day than by night. Does this help to explain the diurnal retinomotor rhythm? The work of Bennitt (1924, 1932*b*) bears upon this problem. Bennitt demonstrated—and the present experiments have confirmed—that under conditions of anaesthesia or of oxygen deficiency there is a migration of pigment towards the light-adapted state, even if the animal is kept in the dark. Anaesthesia and oxygen deficiency are likely to be associated with reduced metabolic rate. Thus it appears that if the metabolic rate is lowered, either in the course of the natural diurnal rhythm or by experimental interference, then there is a tendency for the pigment to migrate towards the light-adapted state, even in the dark. This evidence suggests that the diurnal retinomotor rhythm may be explained as an inevitable consequence of the diurnal metabolic rhythm.

SUMMARY

The demonstration of the existence of a diurnal rhythm in the migration of the pigment in *Leander*, *Portunus*, *Pachygrapsus* and *Carcinus* justifies the supposition that in many species of Crustaceans such a rhythm exists in the retinomotor changes of one of the pigment systems.

These periodic changes in the position of the pigment, despite the exclusion of all light, correspond with sunrise and sunset. Even if the animal is kept in complete darkness during the hours of daytime, the pigment systems concerned exhibit a partial migration towards the position of light-adaptation; when the night returns the pigment system resumes its maximum state of dark-adaptation and is found in the same position as in the previous night.

The measurement of the size of the pseudo-pupil is of value in proving, *in vivo*, the existence of a diurnal rhythm in the iris pigment. The existence of a periodic change in the luminosity of the living eye when examined with the ophthalmoscope is of no value in ascertaining the occurrence of a diurnal rhythm in the retinal pigment or in the tapetum.

In *C. maenas* it was possible to demonstrate a periodic variation in metabolism, in terms of a diurnal rhythm in the production of carbon dioxide. This production was about 30 % smaller during the day than during the night.

The diurnal retinomotor rhythm may depend on a periodic hypoxaemia of the cell systems concerned.

I wish to express my thanks to Prof. J. Jongbloed and Dr A. Punt of the Physiological Laboratory at the University of Utrecht for their kind help and advice in the use of the diaferometer.

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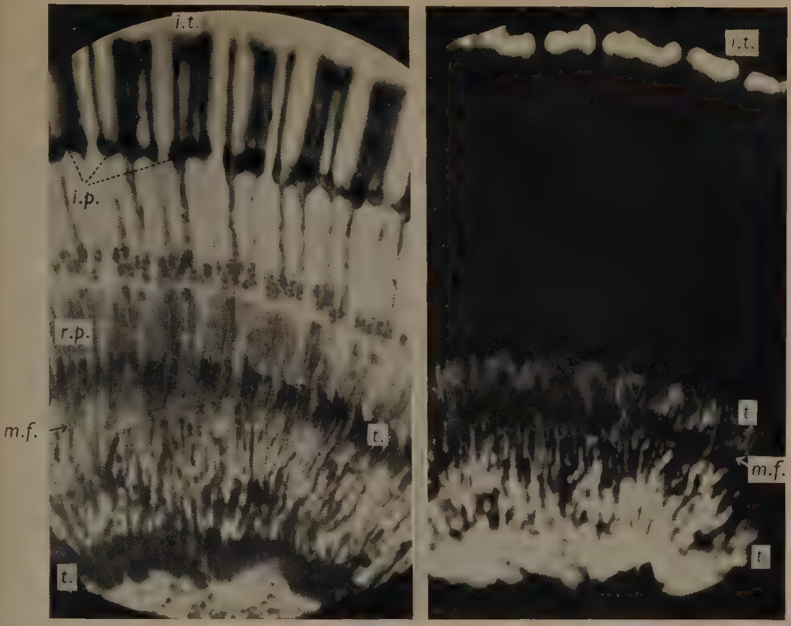

a b

Fig. 1

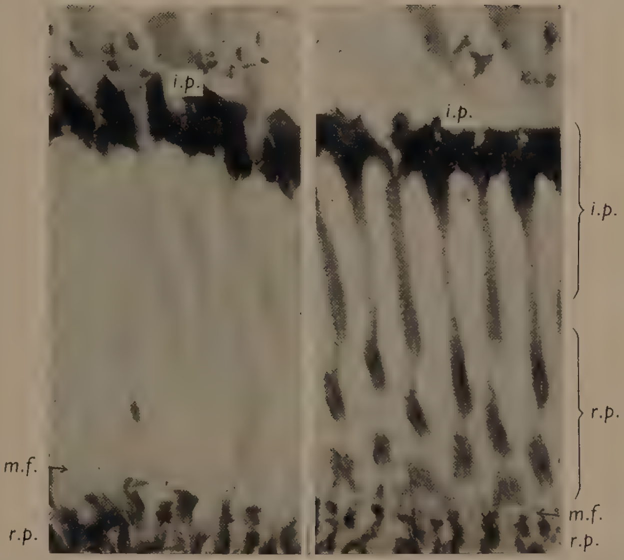

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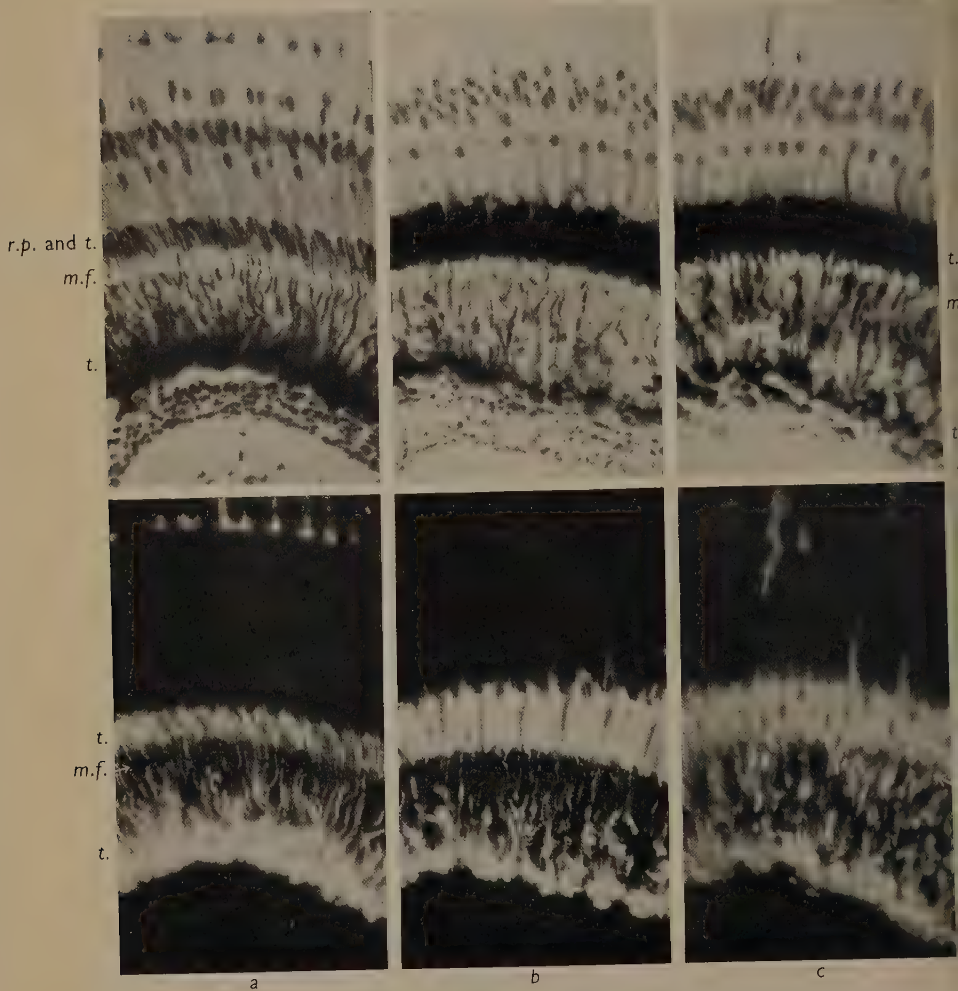
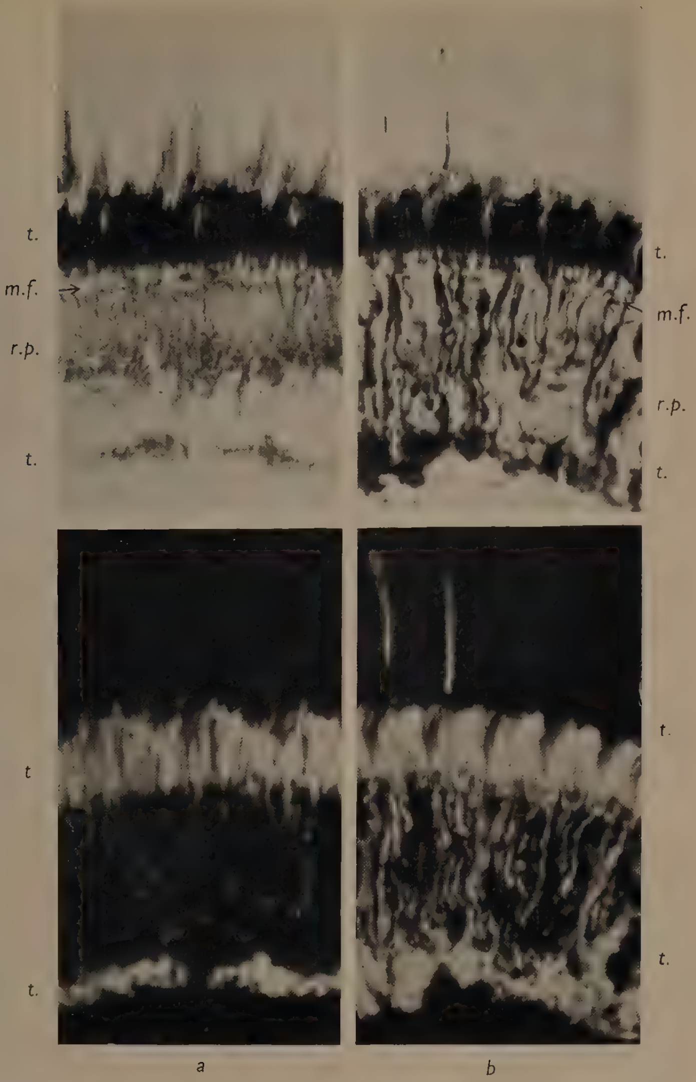
Fig. 2

HENKES—RETINOMOTOR AND DIURNAL RHYTHM
IN CRUSTACEANS



HENKES—RETINOMOTOR AND DIURNAL RHYTHM IN CRUSTACEANS



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EXPLANATION OF PLATES

Plate 6

Fig. 1. Microphotograph of a section through the compound eye of *Leander elegans* in the light-adapted state: (*a*) with normal illumination; (*b*) with dark-ground illumination. Compam (*a*) with Text-fig. 2*a*, left half. In dark-ground illumination only the tapetum and iris tapeture are demonstrable. For abbreviations see Text-figs 1 and 2.

Fig. 2. Diurnal rhythm in the eye of *Carcinus maenas*. (*a*) shows the dark adapted eye during the night; (*b*) shows the dark adapted eye in day-time. Note the marked migration of the retinal pigment in darkness; in day-time it moves between the ommatidia in a distal direction and comes in apposition with the iris pigment ('day-dark position'). No tapetum can be demonstrated.

Plate 7

Micro-photographs of the compound eye of *Leander squilla* in normal illumination and under dark-ground illumination: (*a*) in light-adaptation: (*b*) in dark-adaptation during the night ('night-darkness'); (*c*) in dark-adaptation during the day ('day-darkness'). Note that in (*c*) the tapetum is shifted proximally in the direction of light-adaptation (*a*).

Plate 8

Photographs showing the diurnal rhythm in the compound eye of *Leander elegans* in normal and dark-ground illumination. Note the marked difference in the position of the tapetum between (*a*) 'night-darkness' and (*b*) 'day-darkness'.

PHARMACOLOGICAL INVESTIGATION OF THE NATURE OF THE INNERVATION OF THE MUSCLES OF *HELIX ASPERSA*

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(With Two Text-figures)

Bacq (1947) has reviewed the known actions of autonomic effector substances on invertebrates. Concerning the muscles of snails relatively little is known. Boyer (1926) showed that adrenaline had no effect on the contraction of the ventricular muscle of *Helix pomatia* in a concentration of less than 1 in 20,000, but that higher concentrations slowed the heart and produced irregularities of rhythm. Very high concentrations (1 in 250) arrested the heart beat. According to Jullien (1936) the ventricle of the same snail is sensitive to acetylcholine in a concentration of 1 part per million. There seems to be little other information available, other than the general statements of Bacq (1947) that in many Molluscs physostigmine (eserine) fails to potentiate the effects of acetylcholine, that in Cephalopods curare, but not atropine, abolishes the effect of stimulating the vesical nerve, and that in Molluscs as a whole the muscles of locomotion do not appear to be cholinergic in type.

In the common English snail, *Helix aspersa*, a portion of the alimentary canal (crop and rectum), or of the heart (ventricle), or the columellar muscle (which retracts the main part of the snail within its shell) was isolated, and the effects of drugs upon it examined, with the aim of determining the nature of the neurohormonal mechanism involved in contraction.

METHOD

Large snails were collected and kept in a cool vessel upon fresh leaves. When a snail was required the shell was opened, the foot pinned out, and the columellar muscle, the cardiac ventricle, the rectum or the crop excised. The latter organ was freed of salivary glands, and care was taken to remove it caudal to the ganglia so as to obtain a nerve-free preparation. The organ was suspended between fine nickel hooks in a bath at 18° C., oxygenated, and containing a fluid of the following composition (after Boyer, 1926): $NaCl$ 6·5, KCl 0·14, $CaCl_2$ 0·12, $NaHCO_3$ 0·01, NaH_2PO_4 0·01, in g. per litre. One end of the tissue was attached to a gimbal lever recording on a kymograph. Drugs were added in solution in the nutrient fluid. Histological preparations were made of the organs used.

RESULTS

The fibres of the columellar muscle and in the heart and the alimentary canal resembled vertebrate smooth muscle in general structure. The results obtained after addition of adrenaline HCl, ephedrine HCl, acetylcholine Br, physostigmine salicylate, atropine SO_4, D-tubocurarine, nicotine tartrate, barium Cl_2, magnesium Cl_2, and veratrine are shown in Table 1.

Table 1. *Actions of drugs on muscle from* Helix aspersa

(+ means contraction, − means relaxation, o means no observed effect with a concentration of 10^{-3}, . . . means drug not tested.)

Drug, minimal effective conc. and action	Preparation			
	Columellar muscle	Crop	Rectum	Ventricle
Adrenaline	$10^{-6}-10^{-7}$ +	10^{-6} +	10^{-4} +	10^{-5} slow
Ephedrine	10^{-5} +	o	o	10^{-4} slow and +
Acetylcholine	10^{-6} +	10^{-3} +	10^{-4} +	o
A.Ch. after eserine	10^{-7} +	$2\cdot10^{-4}$ +	$2\cdot10^{-5}$ +	o
A.Ch. after atropine	o	o	o	o
A.Ch. after D-T.B.C.	10^{-6} +	. . .	. . .	. . .
Nicotine	o	o	o	o
Barium	10^{-4} +	10^{-4} +	10^{-4} +	10^{-4} +
Magnesium	10^{-5} −	10^{-4} −	10^{-4} −	. . .
Veratrine	10^{-5} +	10^{-4} +	10^{-4} +	. . .

Potentiation of the effects of acetylcholine following pre-treatment with eserine was clearly visible in the columellar muscle preparation, and to a lesser extent with the crop and rectum and is illustrated in Fig. 1. Abolition of the response to acetylcholine after pre-treatment with atropine occurred if the latter compound was left in contact with the muscle for a prolonged period (10–20 min.) in a concentration of 10^{-6} but not if the acetylcholine was added only 2 or 3 min. after the atropine. At 10^{-5} atropine was quickly effective. Curare (10^{-5} for 20 min.) failed to affect the action of acetylcholine.

Acetylcholine had no effect on the ventricle, but it contracted the crop and rectum (weak action), and this effect also was potentiated by eserine, and reduced or abolished by atropine. Adrenaline had a stimulant action on the columellar muscle, stimulated the rectum and crop (weak action), and slowed the rate of contraction of the ventricle (weak action). These effects were not potentiated by cocaine. Ephedrine also contracted the columellar muscle and affected the ventricle, producing a slower beat and a larger individual contraction. Nicotine exerted no effect on the preparations, which were free of ganglia. The two ions, Ba and Mg, which have a direct action on muscle, had the same effects as in vertebrate preparations, and veratrine caused its customary spasmodic contraction. This, however, was reversible after washing (see Fig. 2). The stimulation of the columellar muscle with cholinergic and sympathomimetic drugs was constant and reproducible, as was that with Ba^{++} and veratrine.

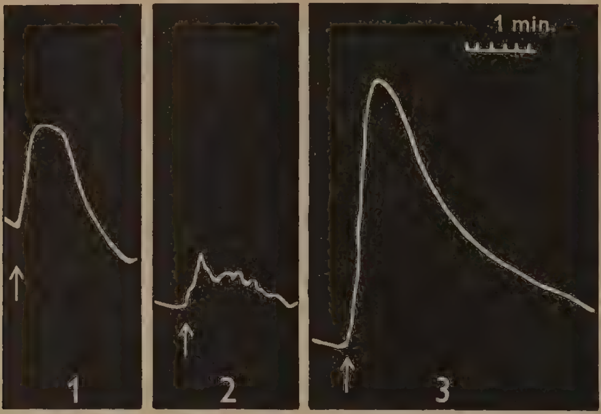

Fig. 1. Isolated columellar muscle of *Helix aspersa*. Acetylcholine 10^{-6} causes contraction (1) which is potentiated (3) by the previous addition (2) of eserine 10^{-5}.

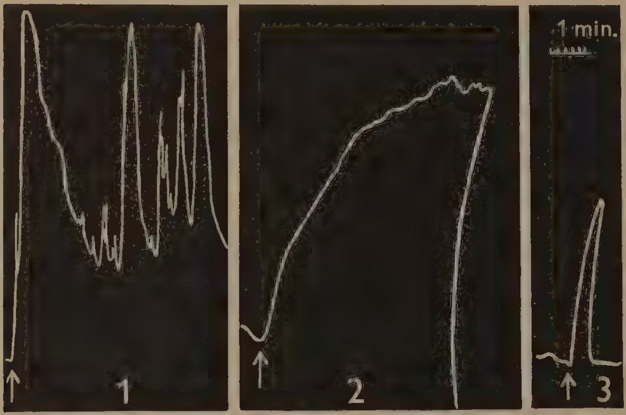

Fig. 2. Isolated columellar muscle of *Helix aspersa*. Adrenaline 10^{-6} causes a repetitive contraction (1). Veratrine 10^{-5} causes a spasm which is reversible on washing (2). Barium 10^{-4} causes a contraction (3).

DISCUSSION

The motor response of the columellar muscle to adrenaline and acetylcholine is curious, but has been observed in higher forms of life (intestine of duck, uterus and ileum of guinea-pig under certain conditions, nictitating membrane of cat). The explanation in the snail may lie in the nature of the response to the two drugs, which differs in detail. The reaction to added adrenaline is a vigorous contraction of rapid onset and short duration, sometimes repetitive in nature. Acetylcholine, on the other hand, usually causes a prolonged spasm. These two types of reaction may be related to differences in function. The potentiation of the actions of acetylcholine by physostigmine suggests that the tissue contains cholinesterase which acts as the normal mechanism of destruction of acetylcholine, and the action of atropine in dilute solution (10^{-6}) in abolishing the action of acetylcholine tends to confirm the supposition that this muscle has a cholinergic muscarinic innervation like much of the smooth muscle of vertebrates. Nevertheless, the properties as well as the appearance of the muscle differ markedly from that of vertebrate smooth muscle. The evidence for cholinergic innervation of the alimentary canal is much less strong since acetylcholine only acts on muscles from this site in the 'unphysiological' concentration of 10^{-4} or more, and its effects are much less clear cut than on the columellar muscle. Nevertheless, physostigmine potentiates these actions, which are abolished by atropine in large doses. No evidence of cholinergic innervation of the heart could be obtained, but by contrast the sympathomimetic compounds adrenaline and ephedrine affected it. They caused a slowing of rhythm, and in the case of ephedrine an increased vigour of contraction. The effects of adrenaline on the preparations from the alimentary canal were too weak and uncertain for any firm conclusion to be drawn, but it is doubtful if any 'autonomic innervation' as we know it in vertebrates is present. The absence of any effect from adding nicotine is not surprising since the preparation is not sensitive to the more potent substances adrenaline and acetylcholine, and is thought to be free of nerve cells. The spasm caused by veratrine was surprisingly easily washed out, in contrast with that seen after veratrine poisoning of vertebrate striped muscle.

SUMMARY

The isolated columellar muscle of *Helix aspersa* is caused to contract by addition to it of ephedrine, adrenaline, veratrine, Ba^{++} and acetylcholine.

Adrenaline and acetylcholine are active in concentrations of 10^{-6} or less.

The action of acetylcholine is potentiated by eserine and abolished by atropine, but not by curare, indicating a muscarinic cholinergic innervation of this muscle.

The heart muscle is sensitive to sympathomimetic substances only.

The muscle of the alimentary canal is insensitive to drugs.

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A COMPARATIVE STUDY OF THE LIPIDS OF THE VERTEBRATE CENTRAL NERVOUS SYSTEM

I. BRAIN

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Previous studies from this laboratory have shown that the distribution of lipids in the brains of a number of different mammalian species is very similar (Johnson, McNabb & Rossiter, 1948). Evidence was presented for the view that free cholesterol and the two sphingosine-containing lipids,* cerebroside and sphingomyelin, are the principal lipids of the myelin sheath of a mammalian nerve fibre (Johnson, McNabb & Rossiter 1949*a*, *b*, 1950; Burt, McNabb & Rossiter, 1950). These lipids were called the myelin lipids. However, McColl & Rossiter (1950, 1951) showed that the lipids of the nervous system of certain invertebrates, notably *Loligo*, *Limulus* and *Libinia*, differed in a number of respects from those of the mammal.

In this paper, data are presented for the lipids of the brains of a series of vertebrates, including representatives of the cartilaginous and bony fishes, amphibians, reptiles, birds and mammals.

METHODS

The brain was dissected from each specimen as soon as possible after death, rapidly weighed, and stored in ethanol until it was convenient to extract and estimate the lipids. For most species the analyses were done on the whole brain, but for some of the mammals the brain was divided down the mid-line and one half only was used. Additional samples were taken for the determination of the wet weight to dry weight ratio, by drying to constant weight at 105° C.

SPECIES STUDIED

The following species were studied:

(1) Class Elasmobranchii (Chondrichthyes)
  Order Euselachii
    I. *Squalus acanthias* Linnaeus (dogfish)
    II. *Caracharias littoralis* Mitchell (sand shark)
    III. *Raia erinacea* Mitchell (common skate)
    IV. *Dasyatis* sp. (sting ray)

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* Carter, Haines, Ledyard & Norris (1947) suggested the name sphingolipid for lipids containing the base sphingosine (i.e. cerebroside and sphingomyelin).

(2) Class Actinopterygii
    Order Holostei
        I. *Amia calva* Linnaeus (bowfin)
    Order Teleostei
        I. *Brevoortia tyrannus* Latrobe (menhaden)
        II. *Perca flavescens* Mitchell (yellow perch)
(3) Class Amphibia
    Order Anura
        I. *Rana pipiens* Schreber (leopard frog)
(4) Class Reptilia
    Order Chelonia
        I. *Clemmys guttata* Schneider (spotted turtle)
(5) Class Aves
    Order Columbiformes
        I. *Columba livia* var. *domestica* Linnaeus (pigeon)
    Order Charadriiformes
        I. *Larus argentatus* Brünnich (herring gull)
(6) Class Mammalia
    Order Rodentia
        I. *Cavia porcellus* Linnaeus (guinea-pig)
    Order Lagomorpha
        I. *Lepus cuniculus* Linnaeus (rabbit)
    Order Carnivora
        I. *Felis domestica* Schreber (domestic cat)

The marine forms were obtained at the Marine Biological Laboratory, Woods Hole, during the summer of 1949, through the courtesy of Dr J. S. Rankin, Summer Naturalist. The fresh-water fish were obtained from Lake Erie. The remaining species were supplied by commercial dealers.

Analytical methods

The lipids were extracted and the concentration of total phospholipin, free and total cholesterol, cerebroside (glycosphingoside), monoaminophospholipin (phosphoglyceride) and lecithin (phosphatidyl choline) was determined in each specimen. From these figures was calculated the concentration of ester cholesterol, sphingomyelin (phosphosphingoside) and kephalin. Details of the analytical procedures may be found in the papers of Johnson *et al.* (1948, 1949*b*). The methods are the same as those used in parallel studies on the lipids of the invertebrate nervous system (McColl & Rossiter, 1950, 1951). The analyses were done either in duplicate or in triplicate.

Accuracy and specificity of analytical methods

The accuracy and specificity of the methods has been discussed previously (Johnson *et al.* 1949*b*; McColl & Rossiter, 1950, 1951). In general, the methods are reasonably specific for the substances for which they were designed. Since little work has been done on the isolation and characterization of lipids from the nervous system of the lower vertebrates, it is possible that lipids, as yet unidentified but with properties similar to those in the mammalian nervous system, might be responsible for some of the values reported. However, Dorée (1909), who isolated cholesterol from a number of vertebrate species, concluded that cholesterol was always present and was not accompanied by any closely related substance. Also Argiris (1908) isolated cerebroside from bird brain and found it to resemble cerebroside isolated from the brain of a mammal. In describing the lipids of the lower vertebrates it is convenient to retain, with the above reservations in mind, the usual lipid nomenclature.

RESULTS

Table 1 shows the mean concentration (± standard deviation) of water, cerebroside, total cholesterol and total phospholipin in the brains of each of the species studied. Table 2 gives similar data for sphingomyelin, lecithin, kephalin and myelin lipid

Table 1. *Mean concentration of water, cerebroside, total cholesterol and total phospholipin in the brain of a representative series of vertebrates*

(The figures in brackets represent the number of individuals studied in each species.)

Species	Mean concentration of lipid in mg. per 100 mg. dry weight (± standard deviation)			
	Water	Cerebroside	Total cholesterol	Total phospholipin
Elasmobranchii:				
Dogfish	4·4 ± 0·43 (4)	7·0 ± 0·53 (6)	6·5 ± 0·57 (6)	20·5 ± 0·55 (6)
Sand shark	4·3 ± (1)	6·4 ± 0·76 (2)	5·8 ± 0·07 (2)	19·6 ± 0·07 (2)
Skate	3·3 ± 0·23 (2)	8·6 ± 0·45 (3)	9·9 ± 0·73 (4)	22·8 ± 0·54 (4)
Sting ray	4·5 ± 0·17 (3)	7·2 ± 0·55 (4)	5·0 ± 0·30 (4)	16·0 ± 0·61 (4)
Class mean (± S.E.M.)	4·1 ± 0·28	7·3 ± 0·47	6·8 ± 1·08	19·7 ± 1·41
Actinopterygii:				
Bowfin	2·9 ± 0·09 (3)	2·7 ± 0·24 (6)	3·5 ± 0·15 (6)	9·4 ± 0·38 (6)
Menhaden	3·2 ± 0·07 (4)	5·9 ± 0·33 (6)	9·7 ± 0·17 (6)	29·4 ± 0·57 (5)
Perch	4·6 ± 0·11 (3)	1·7 ± 0·37 (4)	7·3 ± 0·19 (6)	23·5 ± 0·30 (6)
Class mean (± S.E.M.)	3·6 ± 0·52	3·4 ± 1·27	6·8 ± 1·79	20·8 ± 5·93
Amphibia:				
Frog	5·3 ± 0·13 (3)	6·3 ± 0·76 (6)	5·0 ± 0·19 (6)	18·9 ± 0·30 (6)
Reptilia: Turtle	5·1 ± 0·18 (3)	7·3 ± 0·33 (6)	8·5 ± 0·29 (7)	23·8 ± 0·36 (7)
Aves:				
Pigeon	3·7 ± 0·05 (3)	6·1 ± 0·38 (6)	7·1 ± 0·10 (6)	20·6 ± 0·20 (6)
Gull	4·2 ± 0·03 (3)	7·3 ± 0·12 (5)	6·8 ± 0·07 (5)	20·3 ± 0·07 (5)
Class mean (± S.E.M.)	3·9 ± 0·26	6·7 ± 0·60	6·9 ± 0·16	20·4 ± 0·16
Submammalia:				
Mean of all sub-mammalian species (± S.E.M.)	4·0 ± 0·23	6·0 ± 0·62	6·8 ± 0·60	20·4 ± 1·51
Mammalia:				
Guinea-pig	3·7 ± 0·08 (3)	10·3 ± 0·36 (8)	8·5 ± 0·21 (10)	23·0 ± 0·53 (10)
Rabbit	4·2 ± 0·14 (3)	12·5 ± 0·54 (7)	10·9 ± 0·33 (7)	26·0 ± 0·54 (7)
Cat	3·6 ± 0·13 (3)	11·5 ± 0·65 (20)	10·1 ± 0·34 (20)	22·5 ± 0·59 (20)
Class mean (± S.E.M.)	3·8 ± 0·19	11·4 ± 0·64	9·8 ± 0·71	23·8 ± 1·10

(i.e. the sum of the concentrations of cerebroside, cholesterol and sphingomyelin). The tables also give the class mean (± standard error of mean) for each of the vertebrate classes and the mean for the eleven lower (submammalian) vertebrate species grouped together. Since the concentration of water in the brains was found

Table 2. *Mean concentration of sphingomyelin, lecithin, kephalin and myelin lipid in the brain of a representative series of vertebrates*

(The figures in brackets represent the number of individuals studied in each species.)

Species	Mean concentration of lipid in mg. per 100 mg. dry weight (± standard deviation)			
	Sphingomyelin	Lecithin	Kephalin	Myelin lipid
Elasmobranchii:				
Dogfish	3·5 ± 0·60 (6)	6·5 ± 0·54 (6)	10·8 ± 0·56 (6)	17·0 (6)
Sand shark	3·4 ± 0·31 (2)	6·9 (1)	14·8 (1)	15·6 (2)
Skate	5·6 ± 0·39 (4)	6·5 ± 0·40 (4)	11·2 ± 0·50 (4)	24·1 (4)
Sting ray	2·0 ± 0·51 (4)	5·5 ± 0·36 (4)	8·3 ± 0·53 (4)	14·2 (4)
Class mean (±S.E.M.)	3·6 ± 0·74	6·3 ± 0·30	11·3 ± 1·33	17·7 ± 2·20
Actinopterygii:				
Bowfin	0·5 ± 0·16 (6)	3·5 ± 0·49 (6)	5·5 ± 0·39 (6)	6·7 (6)
Menhaden	2·9 ± 0·19 (5)	10·9 ± 0·57 (4)	13·8 ± 0·61 (4)	18·5 (5)
Perch	5·2 ± 0·58 (6)	8·4 ± 0·25 (6)	9·5 ± 0·24 (6)	14·2 (6)
Class mean (±S.E.M.)	2·9 ± 1·36	7·6 ± 2·17	9·6 ± 2·40	13·1 ± 3·45
Amphibia:				
Frog	2·0 ± 0·54 (6)	8·2 ± 0·18 (6)	7·6 ± 0·44 (6)	13·3 (6)
Reptilia:				
Turtle	2·7 ± 0·26 (7)	7·9 ± 0·27 (7)	13·4 ± 0·24 (7)	18·5 (6)
Aves:				
Pigeon	3·3 ± 0·27 (6)	6·1 ± 0·10 (6)	11·3 ± 0·17 (6)	16·5 (6)
Gull	2·3 ± 0·44 (5)	6·8 ± 0·29 (5)	12·0 ± 0·19 (5)	16·4 (5)
Class mean (±S.E.M.)	2·8 ± 0·50	6·4 ± 0·35	11·6 ± 0·35	16·4 ± 0·07
Submammalia:				
Mean of all submammalian species (±S.E.M.)	3·0 ± 0·44	6·9 ± 0·58	10·7 ± 0·85	15·9 ± 0·90
Mammalia:				
Guinea-pig	4·4 ± 0·21 (8)	6·6 ± 0·10 (2)	13·2 ± 0·15 (2)	23·2 (8)
Rabbit	4·5 ± 0·32 (7)	7·3 ± 0·18 (7)	14·6 ± 0·30 (7)	27·9 (7)
Cat	4·3 ± 0·27 (20)	6·0 ± 0·21 (20)	12·4 ± 0·45 (20)	25·9 (20)
Class mean (±S.E.M.)	4·4 ± 0·06	6·6 ± 0·38	13·4 ± 0·98	25·7 ± 1·36

to vary considerably from species to species, the results are given in terms of unit dry weight, rather than in terms of wet weight. As was found previously for the nervous system of both mammals and invertebrates, the concentration of cholesterol ester was negligibly small and has not been recorded. Unlike certain invertebrates, such as *Limulus* (McColl & Rossiter, 1950) and *Loligo* (McColl & Rossiter, 1951), cerebroside was detected in all the vertebrate brains, although in some species the concentration was rather small.

The small standard deviations for the concentration of the lipids in the brain of most species indicate that, despite a considerable species-to-species variation, the concentration of each lipid in the brains of different individuals of the same species is quite similar. A detailed statistical analysis of all the data has not been undertaken, but it is apparent that in many instances the differences between the mean values for two species within the same class are greater than the differences between class means. For example, it is obvious that for almost every lipid the difference between the means for any two of the three actinopterygian species is significant statistically. On the other hand, with the exception of cerebroside, for none of the lipids is the class mean for actinopterygians significantly different from the class mean for corresponding lipid in the elasmobranchs.

Table 3. *Values of P for class-mean data presented in Tables* 1 *and* 2. *Each column gives the values of P obtained in testing the significance of the difference between the means for that particular class and the class means for the mammal. P obtained by the Fisher* '*t*' *test*

	Elasmobranchii	Actinopterygii	Amphibia	Reptilia	Aves	All submammalia
Water	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Cerebroside	<0·01	<0·01	N.S.	N.S.	<0·02	<0·01
Total cholesterol	N.S.	N.S.	N.S.	N.S.	<0·05	<0·05
Total phospholipin	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Sphingomyelin	N.S.	N.S.	<0·01	<0·01	<0·05	N.S.
Lecithin	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Cephalin	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Myelin lipid	<0·05	<0·05	<0·05	N.S.	<0·02	<·010

(N.S., not significant, i.e. $P>0·05$.)

Because of the small number of species studied and the wide range in the mean lipid concentration for the different species within each class, few generalizations are possible from these data. Table 3 gives the value of P obtained when the significance of the difference between any class mean and the class mean for the mammals is tested by Fisher's 't' test. For water, total phospholipin, lecithin and kephalin, none of the class means differs significantly from that of the corresponding lipid for the mammals, nor does the mean of all of the eleven submammalian species grouped together. Cerebroside, total cholesterol, sphingomyelin and myelin lipid are the only lipids for which any of the differences are statistically significant ($P<0·05$). For cerebroside the mean of all the submammalian species grouped together and the class means of the actinopterygians, elasmobranchs and birds are significantly different from the class mean of the mammals. For total cholesterol the means of the birds and all the submammalian species grouped together differ significantly from the class mean of the mammals, whereas for sphingomyelin all the class means, with the exception of those of the two classes of fishes, differ significantly from those of the mammals. A significant difference in the concentration of the myelin lipid was to be anticipated, since the concentration of myelin lipid represents the sum of the concentrations of cerebroside, cholesterol and sphingomyelin, the three individual lipids for which the differences are significant.

DISCUSSION

The figures presented here for the concentration of lipids in the mammalian brain are in accord with those reported by other workers (Johnson *et al.* 1948, for references).

Comparative studies on the lipids of vertebrate brain have been rare. Such reports as are available indicate that mammalian brain is distinguished from the brains of lower vertebrates in containing a greater concentration of cerebroside and cholesterol. There is no significant difference in the concentration of total phospholipin. Argiris (1908) found a low concentration of cerebroside in the brain of the codfish, a result confirmed by Lanfranchi (1938), who reported that the concentration of cerebroside in the brain of certain vertebrates increases with evolutionary development. Our figures for the actinopterygians, reptiles, birds and mammals agree with the results of Lanfranchi (1938), but those for the elasmobranchs and amphibians are considerably greater. However, in a general way, our figures support the conclusion of Lanfranchi (1938). Rosenheim (1914), Koch & Riddle (1919) and Gassner (1927) showed that the concentration of cholesterol in the brain of the bird and the fish is less than that in the brains of a representative series of mammals, whereas Koch & Riddle (1919) and Javillier, Crémieu & Hinglais (1928) reported that the concentration of total phospholipin in the brain of the bird and dogfish is of the same order as that in mammalian brain. Our figures for the dogfish and bird brain agree with those of the above workers. We are aware of no previous comparative study on the lipids of brain that included the determination of the individual phospholipins.

The mammalian brain differs from the brain of the lower vertebrates in that it contains a greater concentration of cerebroside, cholesterol and sphingomyelin. These are the three substances that are thought to be the principal lipid components of the myelin sheath of a mammalian nerve fibre (Johnson *et al.* 1950; Burt *et al.* 1950, for references). The white matter of mammalian brain, which contains many myelinated nerve fibres, contains a higher concentration of these three lipids than the grey matter, which contains most of the nerve cell bodies and comparatively few myelinated nerve fibres. On the other hand, there is little difference in the concentration of lecithin and kephalin (Johnson *et al.* 1949*a*). The finding that cerebroside, cholesterol and sphingomyelin, and not lecithin and kephalin, occur in a higher concentration in the brains of mammals than in the brains of lower vertebrates is capable of two interpretations, either or both of which may be true:

(1) The chemical nature of the lipids of the 'myelin' in the brains of the lower vertebrates differs from that in mammalian brain.

(2) The relative amount of 'myelin' material in the mammalian brain is greater than that in the brains of the lower vertebrates.

Since we are aware of no available data on the relative amounts of white matter and grey matter in the brains of different vertebrate species, we have been unable to choose between these alternatives.

SUMMARY

1. The concentration of water, cerebroside (glycosphingoside), free and total cholesterol, total phospholipin, monoaminophospholipin (phosphoglyceride) and lecithin (phosphatidyl choline) was determined in the brains of a series of vertebrates, including representatives of the cartilaginous and bony fishes, amphibians, reptiles birds and mammals. From these figures was calculated the concentration of ester cholesterol, sphingomyelin (phosphosphingoside) and kephalin.

2. The brains of each of the species contained a negligibly small concentration of cholesterol ester and, unlike the nervous system of certain invertebrates, the brains of all the species contained some cerebroside.

3. The concentration of each lipid in the brain was very similar for different individuals of the same species, but for various species within one class the mean concentration of many of the lipids differed greatly.

4. The mean concentration of total myelin lipid and that of each of the individual myelin lipids, i.e. cerebroside, cholesterol and sphingomyelin, was greater in the brains of the mammals than in the brains of the lower vertebrates.

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A COMPARATIVE STUDY OF THE LIPIDS OF THE VERTEBRATE CENTRAL NERVOUS SYSTEM

II. SPINAL CORD

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(With One Text-figure)

INTRODUCTION

In the preceding paper McColl & Rossiter (1952) reported the concentration of water, cerebroside, total cholesterol, total phospholipin, sphingomyelin, lecithin, kephalin and myelin lipid in the brains of a representative series of vertebrates. This paper describes the distribution of these substances in the spinal cords of the same series of vertebrates.

METHODS

The species studied and the analytical methods were the same as those described in the previous paper (McColl & Rossiter, 1952). Samples of spinal cord were removed from each specimen as soon as possible after death. The sample was usually taken from the mid-dorsal region, although in some of the smaller species the entire dorsal cord was used.

RESULTS

Table 1 shows the mean concentration (± standard deviation) of water, cerebroside, total cholesterol and total phospholipin, and Table 2 the mean concentration of sphingomyelin, lecithin, kephalin and myelin lipid for each of the species studied.

These tables can be compared with Tables 1 and 2 of the preceding paper. The figures are all given in terms of unit dry weight. In almost every instance the concentration of water is less, and the concentration of cerebroside, total cholesterol, total phospholipin, sphingomyelin and myelin lipid is greater in the spinal cord than in the brain. Lecithin and kephalin are more variable, the concentration in the brain at times exceeding that in the spinal cord.

As was found for the brain, the concentration of each lipid is very similar for different individuals of the same species, but the mean concentrations of many of the lipids extend over a wide range for the different species within one class. The concentration of ester cholesterol was found to be negligibly small and is not recorded.

Tables 1 and 2 also give the class mean (± standard error of mean) for the mammals, and the mean for all of the submammalian species grouped together. The significance of the differences between these two means is also given in the tables.

Although the differences for both myelin lipid (sum of the cerebroside, cholesterol and sphingomyelin) and cholesterol are significant statistically, the figures for cerebroside and sphingomyelin are not. It is of interest to note, however, that the difference for sphingolipid (which represents the sum of the cerebroside and sphingomyelin) is significant. This is due to the inverse relation between the concentrations of cerebroside and sphingomyelin to be discussed below.

Table 1. *Mean concentration of water, cerebroside, total cholesterol and total phospholipin in the spinal cords of a representative series of vertebrates*

(The figures in brackets represent the number of individuals studied in each species.)

Species	Mean concentration of lipid in mg. per 100 mg. dry weight (± standard deviation)			
	Water	Cerebroside	Total cholesterol	Total phospholipin
Elasmobranchii:				
Dogfish	2·2 ± 0·30 (3)	15·0 ± 0·74 (6)	12·5 ± 1·31 (5)	25·6 ± 1·11 (6)
Sand shark	2·3 (1)	15·8 ± 0·26 (2)	15·2 ± 0·01 (2)	30·3 ± 0·30 (2)
Skate	2·2 ± 0·05 (3)	14·4 ± 0·25 (4)	14·4 ± 0·48 (4)	31·0 ± 0·20 (4)
Sting ray	2·4 ± 0·07 (3)	14·3 ± 0·51 (4)	12·6 ± 0·33 (4)	30·2 ± 2·42 (4)
Actinopterygii:				
Bowfin	2·9 ± 0·10 (3)	10·9 ± 0·23 (6)	12·9 ± 0·55 (6)	25·4 ± 0·98 (6)
Menhaden	2·0 ± 0·09 (4)	6·3 ± 0·47 (6)	15·3 ± 0·39 (6)	39·3 ± 1·20 (6)
Perch	2·6 ± 0·09 (3)	6·5 ± 0·30 (5)	15·1 ± 0·78 (6)	34·2 ± 1·12 (6)
Amphibia:				
Frog	4·1 ± 0·01 (3)	19·9 ± 0·40 (6)	10·7 ± 0·01 (5)	20·9 ± 0·33 (4)
Reptilia:				
Turtle	2·9 ± 0·25 (3)	7·8 ± 0·87 (6)	14·8 ± 0·49 (6)	27·7 ± 0·79 (6)
Aves:				
Pigeon	2·2 ± 0·10 (3)	14·1 ± 1·10 (7)	12·5 ± 0·58 (7)	18·6 ± 1·06 (7)
Gull	2·4 ± 0·13 (3)	14·3 ± 0·67 (5)	11·9 ± 0·21 (5)	22·1 ± 0·58 (5)
Submammalia:				
Mean of all submammalian species (± S.E.M.)	2·6 ± 0·18	12·7 ± 1·29	13·4 ± 0·47	—
Mammalia:				
Guinea-pig	2·4 ± 0·05 (3)	12·9 ± 1·10 (6)	15·6 ± 0·26 (6)	26·5 ± 0·65 (6)
Rabbit	2·1 ± 0·04 (3)	18·3 ± 0·26 (7)	18·3 ± 0·29 (6)	32·9 ± 0·45 (6)
Cat	2·1 ± 0·04 (3)	19·2 ± 1·20 (7)	18·3 ± 0·53 (7)	28·8 ± 0·86 (7)
Class mean (± S.E.M.)	2·2 ± 0·10	16·8 ± 1·97	17·4 ± 0·90	—
P (difference between means of mammalian and submammalian species)	Not significant	Not significant	$< 0·01$	—

In Table 3 the mean values for the lipids in both the brain and the spinal cord of each species are recorded as a percentage of the concentration of 'essential lipid'. 'Essential lipid' is defined for this purpose as the sum of the cerebroside, total cholesterol and total phospholipin. The figures for brain are calculated from the data presented in Tables 1 and 2 of the previous paper (McColl & Rossiter, 1952) and those for spinal cord from Tables 1 and 2 of this paper. In a number of instances where the percentage of sphingomyelin is low the percentage of cerebroside is high. This point is well illustrated in the actinopterygians. In both the brain and spinal cord of the bowfin (Order Holostei), where the percentage of cerebroside is high, the percentage of sphingomyelin is low. On the other hand, in the brain and the spinal cord of the perch (Order Teleostei), where the percentage of sphingomyelin is high, the percentage of cerebroside is low. In Fig. 1 the mean percentage of cerebroside is plotted against the mean percentage of sphingomyelin for the spinal cord of each of the species. It can be seen that the two lipids are negatively correlated ($r = -0{\cdot}635 \pm 0{\cdot}172$). Each of these two lipids contains the base sphingosine. It would appear that the spinal cords of some species maintain a high concentration of one sphingosine-containing lipid at the expense of the other. Such an inverse relation is less evident in the lipids of brain.

Table 2. *Mean concentration of sphingomyelin, lecithin, kephalin, and myelin lipid in the spinal cords of a representative series of vertebrates*

(The figures in brackets represent the number of individuals studied in each species.)

Species	Mean concentration of lipid in mg. per 100 mg. dry weight (± standard deviation)			
	Sphingomyelin	Lecithin	Kephalin	Myelin lipid
Elasmobranchii:				
Dogfish	6·1 ± 0·55 (6)	7·4 ± 0·62 (6)	12·1 ± 0·62 (6)	33·6 (6)
Sand shark	7·3 ± 0·75 (2)	9·6 (1)	15·5 (1)	38·3 (1)
Skate	8·6 ± 0·45 (4)	9·6 ± 0·17 (4)	12·8 ± 0·58 (4)	37·4 (4)
Sting ray	6·5 ± 0·51 (4)	8·5 ± 1·33 (4)	15·0 ± 1·25 (4)	33·4 (4)
Actinopterygii:				
Bowfin	3·9 ± 0·52 (6)	8·2 ± 0·35 (6)	13·3 ± 0·39 (6)	27·7 (6)
Menhaden	9·3 ± 0·60 (6)	13·8 ± 1·14 (5)	15·0 ± 0·44 (5)	30·9 (5)
Perch	13·7 ± 0·67 (6)	12·6 ± 0·35 (6)	8·3 ± 0·52 (6)	35·3 (6)
Amphibia:				
Frog	5·1 ± 0·33 (4)	8·7 ± 0·25 (4)	7·7 ± 0·79 (4)	35·7 (4)
Reptilia:				
Turtle	7·4 ± 0·64 (6)	7·4 ± 0·37 (6)	12·9 ± 0·62 (6)	30·0 (6)
Aves:				
Pigeon	6·1 ± 0·59 (7)	3·8 ± 0·42 (7)	8·6 ± 0·72 (7)	32·7 (7)
Gull	3·7 ± 0·24 (5)	5·8 ± 0·28 (5)	12·6 ± 0·60 (5)	29·9 (5)
Submammalia:				
Mean of all submammalian species (± S.E.M.)	7·1 ± 0·85	8·7 ± 0·85	12·2 ± 0·84	33·2 ± 1·01
Mammalia:				
Guinea-pig	7·1 ± 0·38 (6)	5·8 ± 0·30 (6)	13·9 ± 0·45 (6)	35·6 (6)
Rabbit	11·5 ± 0·61 (6)	5·6 ± 0·08 (6)	16·7 ± 0·49 (6)	48·1 (6)
Cat	9·6 ± 0·48 (7)	4·7 ± 0·34 (7)	14·9 ± 0·99 (7)	47·1 (7)
Class mean (± S.E.M.)	9·4 ± 1·32	5·4 ± 0·34	15·2 ± 0·82	43·6 ± 4·01
P (difference between means of mammalian and submammalian species)	Not significant	Not significant	Not significant	< 0·01

Table 4 gives an analysis of the results presented in Table 3. In all 14 of the species studied there is a greater percentage of both myelin lipid and sphingolipid in the spinal cord than in the brain. The percentage of cerebroside in the spinal cord exceeds that in the brain in 10 of the 14 (1 not significant) species. For total cholesterol the figure is 13 out of 14 (1 not significant), and for sphingomyelin it is 14 out of 14. On the other hand, for lecithin the percentage of lipid in the brain exceeds that in the spinal cord in 13 of the 14 (1 not significant) species, and for kephalin the figure is 14 out of 14. It is thus apparent that, over the whole range of species studied, the lipids of the spinal cord, as distinct from those of the brain, are characterized by a higher percentage of cerebroside, cholesterol and sphingomyelin, and a lower percentage of lecithin and kephalin.

Table 3. *Lipids of the brains and spinal cords of a representative series of vertebrates expressed as a percentage of 'essential lipid'*

Species	Cerebroside		Total cholesterol		Sphingomyelin		Lecithin		Kephalin	
	Brain	Cord	Brain	Cord	Brain	Cord	Brain	Cord	Brain	Cord
Elasmobranchii:										
Dogfish	20·6	28·3	19·0	23·5	10·3	11·4	19·0	13·8	31·7	22·9
Sand shark	20·0	25·8	18·3	24·7	10·7	11·8	21·7	15·6	46·7	25·2
Skate	20·8	24·1	23·9	24·0	13·5	14·4	15·6	16·0	27·0	21·4
Sting ray	25·5	25·0	17·6	22·0	7·3	11·3	19·6	14·9	29·4	26·2
Actinopterygii:										
Bowfin	17·5	22·0	22·5	26·2	3·0	7·9	22·5	16·7	35·0	27·0
Menhaden	13·1	10·3	21·5	25·1	6·4	15·3	24·3	22·7	30·8	24·6
Perch	5·2	11·6	22·4	27·1	16·0	24·5	25·9	22·6	29·3	14·8
Amphibia:										
Frog	20·8	38·6	16·7	20·8	6·5	9·9	27·1	16·8	25·0	14·9
Reptilia:										
Turtle	18·5	15·5	21·5	29·4	6·9	14·7	20·0	14·7	33·8	25·6
Aves:										
Pigeon	18·1	31·2	20·8	27·6	9·9	12·8	18·1	8·5	33·3	19·1
Gull	20·6	29·6	19·1	24·6	6·5	7·7	19·1	12·0	33·8	26·0
Mammalia:										
Guinea-pig	24·7	23·5	20·2	28·4	10·4	13·0	15·7	10·4	31·4	25·3
Rabbit	25·3	26·3	22·1	26·4	9·1	15·2	14·7	8·0	29·5	24·1
Cat	26·0	29·0	22·9	27·5	9·8	13·5	13·5	7·0	28·1	22·4

Table 4. *Analysis of data presented in Table 3*

Lipid	No. of species	Percentage in cord less than percentage in brain	Percentage in cord greater than percentage in brain	No significant difference
Cerebroside	14	3	10	1
Total cholesterol	14	0	13	1
Sphingomyelin	14	0	14	0
Lecithin	14	13	0	1
Kephalin	14	14	0	0
Myelin lipid	14	0	14	0
Sphingolipid	14	0	14	0

DISCUSSION

It has long been known that for the mammal the spinal cord contains less water and more lipid than the brain (see Halliburton, 1894, and Fraenkel & Linnert, 1910, for references). The results reported here show that the same is true over a wide range of vertebrate species. For certain lipids, however, notably lecithin and kephalin, the difference between brain and spinal cord may not be very great, and in some species the advantage may lie with the brain rather than with the spinal cord. Our results for the lipids of the spinal cord of mammals agree reasonably well with previously published figures, i.e. those of Brante (1949) for cerebroside, cholesterol, total phospholipin, sphingomyelin, lecithin and kephalin, and those of Samuels, Boyarsky, Gerard, Libet & Brust (1951) for total phospholipin.

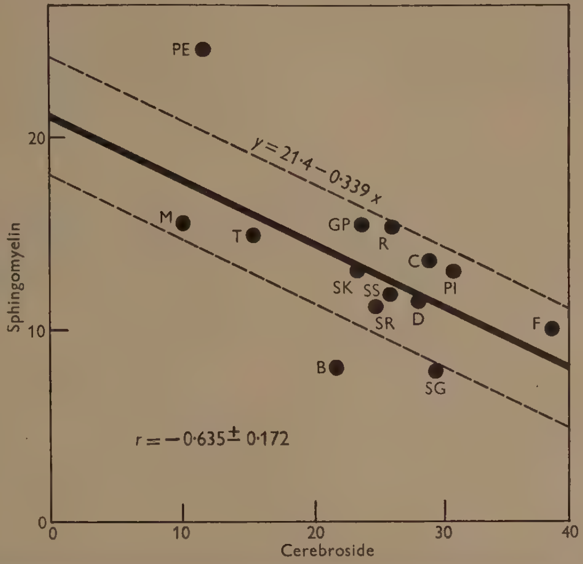

Fig. 1. Relation between the concentration of cerebroside and the concentration of sphingomyelin in the spinal cords of a series of vertebrate species. The figures are expressed as a percentage of 'essential lipid'. D, dogfish; SS, sand shark; SK, skate; SR, sting ray; B, bowfish; M, menhaden; PE, perch; F, frog; T, turtle; PI, pigeon; SG, sea-gull; GP, guinea-pig; R, rabbit; C, cat.

The inverse relation between cerebroside and sphingomyelin emphasizes the usefulness of the term sphingolipid, introduced by Carter, Haines, Ledyard & Norris (1947). These two lipids, which are not usually classified together, have a number of similar physical and chemical properties, and the present findings suggest the possibility that one may replace the other in the structure of the myelin sheath. Such a suggestion was previously made by Sadhu (1948). In this regard it is of interest to note that Williams, Galbraith, Kaucher, Mayer, Richards & Macy (1945) reported that, expressed as a percentage of 'essential lipid', the cerebroside of rat brain increased and the sphingomyelin decreased as the animals became older.

These results go further to establish the view that cerebroside, cholesterol and sphingomyelin, rather than lecithin or kephalin, are the principal lipids in the myelin sheath of a vertebrate nerve fibre. We have called these three lipids the myelin lipids. It is convenient at this time to summarize our reasons for believing that these lipids play an important part in myelin-sheath structure.

(1) The white matter of mammalian brain, which contains many myelinated nerve fibres, has a higher concentration of these three myelin lipids than has grey matter, which contains comparatively few myelinated fibres (Johnson, McNabb & Rossiter, 1948*a*). Peripheral nerve, which is also rich in 'myelin', resembles the white matter of brain rather than the grey matter (Johnson, McNabb & Rossiter, 1948*b*).

(2) Although the distribution of lipids in the grey matter of adult brain is similar to that of the brain of newborn infants, the distribution of lipids in the white matter is very different. The white matter of adult brain, in which myelination is complete, differs from the 'white' matter of the brains of newborn infants, in which myelination is far from complete, in containing a higher concentration of these same three myelin lipids (Johnson, McNabb & Rossiter, 1949*a*).

(3) After section of a peripheral nerve (neurotmesis) the myelin lipids disappear from the degenerating peripheral segment at a similar rate (Johnson, McNabb & Rossiter, 1949*b*, 1950). In a nerve regenerating after a crushing procedure that interrupts the axons and myelin sheath but leaves the connective tissue sheaths intact (axonotmesis) the myelin lipids reappear together (Burt, McNabb & Rossiter, 1950).

(4) The brains of mammals contain a significantly greater concentration of the myelin lipids than do the brains of lower vertebrates (McColl & Rossiter, 1951). This evidence is valid only if it is assumed (*a*) that the brains of mammals contain more 'myelin' than those of the lower vertebrates, and (*b*) that the 'myelin' of the brains of all vertebrates has a similar chemical composition.

(5) It has now been shown that, for a wide variety of vertebrate species, the concentration of these three myelin lipids in the spinal cord is greater than that in the brain. Again, this evidence is valid only if (*a*) the spinal cord contains more 'myelin' than the brain, a reasonable assumption having regard to the microscopic structure and water content of the two tissues, and (*b*) the 'myelin' of the spinal cord of each species has a similar, although not necessarily an identical, chemical composition.

Each of these five lines of evidence indicates that the principal lipids of the myelin sheath of a vertebrate nerve fibre are cerebroside, cholesterol and sphingomyelin. It in no way follows that these three are the only lipids contained in the myelin sheath, but they are probably those present in the greatest quantity and those that distinguish the myelin sheath from other parts of the nervous system. In no instance does the distribution of total phospholipin, lecithin, or kephalin parallel the distribution of myelin. The myelin sheath may contain some lecithin, but the analyses show that considerable quantities of lecithin are also present in other non-myelin structures of the nervous system. The position of kephalin is more equivocal. Recently, Folch (1949) has shown that the 'kephalin' of the nervous system estimated by the methods used in the present study is a mixture of at least three separate lipids: phosphatidyl serine, phosphatidyl ethanolamine and brain diphosphoinositide. The exclusion of 'kephalin' from the myelin lipids does not necessarily exclude any one of the individual constituents of 'kephalin'. In fact, Brante (1949) has published data supporting the view that phosphatidyl serine is a myelin lipid. The position of phosphatidyl ethanolamine and brain disphosphoinositide is not so clear. It is to be hoped that with the development of more satisfactory analytical methods the position of 'kephalin', and of the individual constituents of 'kephalin', will be further clarified.

SUMMARY

1. The concentration of water, cerebroside (glycosphingoside), free and total cholesterol, total phospholipin, monoaminophospholipin (phosphoglyceride) and lecithin (phosphatidyl choline) was determined in the spinal cords of a series of vertebrates including representatives of the cartilaginous and bony fishes, amphibians, reptiles, birds and mammals. From these figures was calculated the concentration of ester cholesterol, sphingomyelin (phosphosphingoside) and kephalin.

2. As was previously found for brain, the spinal cords of each of the species studied contained negligibly small concentrations of cholesterol ester.

3. The concentration of each lipid in the spinal cord was very similar for different individuals of the same species, but for different species within the same class the mean concentration of many of the lipids extended over a wide range.

4. The concentration of total cholesterol, sphingolipid and myelin lipid was greater in the spinal cords of mammals than in the spinal cords of the lower vertebrates.

5. Expressed as a percentage of 'essential lipid', the concentration of cerebroside, cholesterol, sphingomyelin, sphingolipid and myelin lipid, but not of lecithin and kephalin, was greater in the spinal cord than in the brain.

6. For the spinal cords of the species studied, the mean concentration of cerebroside expressed as a percentage of 'essential lipid' was negatively correlated with the mean concentration of sphingomyelin.

7. The results are discussed in relation to the chemical nature of the lipids of the myelin sheath of a vertebrate nerve fibre.

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A ROUNDABOUT FOR STUDYING SUSTAINED FLIGHT OF LOCUSTS

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(With Five Text-figures)

The purpose of the technique described here was to study the influence of external factors upon the flying speed and the ability of small 'swarms' of locusts to sustain prolonged flight. The wind forces acting on a flying animal depend only on the motion of its elements relative to the air mass, i.e. on the relative wind. Instead of investigating actual free flight, a locust can therefore be suspended in front of a wind tunnel and made to fly against a streamlined and uniform current of air, the wind speed being adjusted so as to correspond to the air speed of the freely flying insect. Under such conditions it has been possible to make a single individual fly for several hours. But a very large tunnel would be necessary for a number of animals to fly simultaneously as is required for quantitative work. Instead of moving the air mass, one can move the suspended animal through still air with a speed corresponding to the flying speed and so have several insects flying at once. Thus proper aerodynamic conditions for flight can be obtained by attaching the animals to a revolving ring, provided the ring is made so large that the angular velocity is kept small.

Locusts are well suited for experiments on insect flight, because a suspended locust will move its wings when exposed to an air current (Fraenkel, 1932; Kennedy, Ainsworth & Toms, 1948). This was found to be due to an aerodynamic sense organ consisting of patches of sensory hairs on the head capsule: when these hairs are met by an air flow the animal starts to beat its wings (Weis-Fogh, 1949, 1950). Consequently it was possible to induce wing movements in suspended locusts by revolving them through the air. Kennedy *et al.* (1948) used this idea for studying the uptake of spray drops by flying locusts. The locusts were suspended from the periphery of a small roundabout, 78 cm. in diameter. By means of a gramophone motor the insects were revolved through the air at a constant speed of 2·75 m./sec., while beating their wings. But they were not allowed to choose their own flying speed and, according to flash photographs, the wing movements seemed to be impeded by the abdomen, which was bent outwards. Since our purpose was to study the flight performance of a 'swarm' of locusts under conditions as natural as possible we constructed a roundabout which would allow the insects to fly at a self-chosen speed, the animals being suspended so that they were able to fly unimpeded until exhausted, i.e. for several hours.

APPARATUS AND SUSPENSION

The roundabout should be as large as possible in order to diminish the angular velocity, but for practical reasons we decided upon a revolving horizontal ring of 2 m. diameter (Fig. 1). The ring was suspended by means of eight thin spokes originating from a hub which rested on ball-bearings. The lowest part of the hub was provided with a 16-bladed mill which was driven by means of four jets of air fed from a gas compressor unit. The output of the compressor could be regulated by means of a variable electrical resistance, and so the roundabout could be revolved at various speeds, no mechanical transmission being involved. By means of a sticky wax (Krogh & Weis-Fogh, 1951) the posterior, shield-shaped part of the pronotum of the insect was fixed to a small transverse plate of a suspending bar (Fig. 2). The bar was attached to the ring in such a manner that the locust swung out freely under the centrifugal force. In this way, the resultant of the force of gravity and the centrifugal force acted in the median plane of the animal, so preventing any outward bending of the body. If bent, the pterothorax would be set at an angle to the prothorax, the protruded caudo-lateral margin of which would then touch the base of one of the forewings and impede its movements. The plate of the suspending bar was arranged so that the suspended animal was inclined at about 5–10° to the horizontal plane. This inclination was partly based upon some photographs of desert locusts (*Schistocerca gregaria* Forskål) put at our disposal by Dr D. L. Gunn

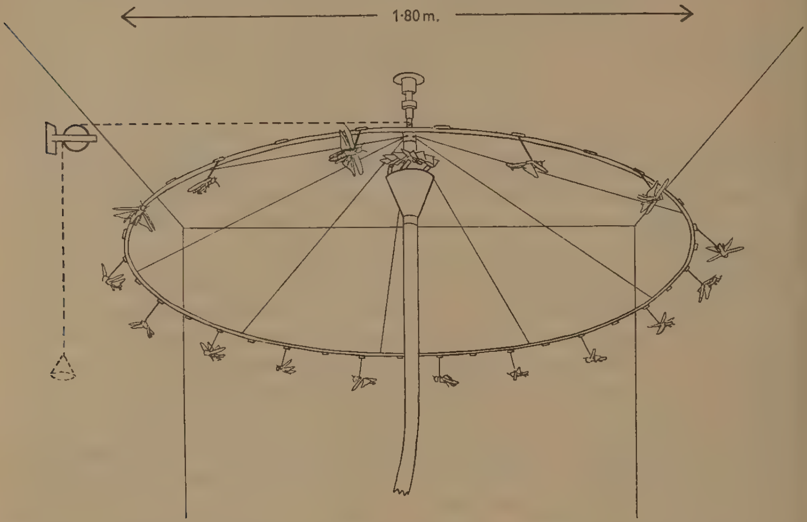

Fig. 1. The revolving roundabout with sixteen desert locusts suspended. One individual (a truant) in the foreground to the left is shown not flying. The suspending bars swing out freely due to the centrifugal force. Drawn from flash photograph, the speed of the periphery (the flying speed) being about 3 m./sec.

and partly on observations on the same species, flying in front of a wind tunnel. When inclined at 5–10° such locusts could be made to fly at a completely normal posture (antennae stretched diagonally forwards, forelegs bent up, middle legs stretched along the abdomen, and hind tibiae bent up under the femura). Relatively small deviations in angle caused the animal to give up the normal posture.

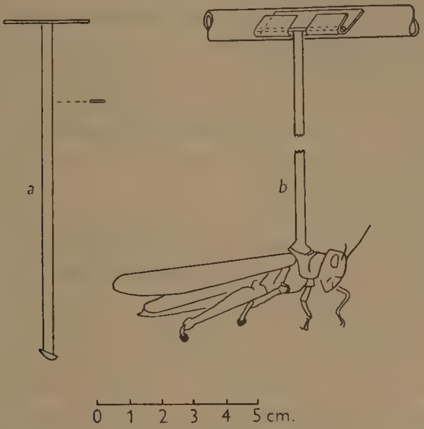

Fig. 2. The suspension of locusts. *a*, suspending bar, cross-section to the right. *b*, a desert locust attached to the roundabout. The end-plate of the suspending bar is sealed to the shield-shaped pronotum by means of sticky wax. The hind-tibiae and the mesotarsi have been cut and the wounds covered with wax: the animal cannot get any contact stimuli which would inhibit the wing movements.

The ring could carry thirty-two locusts at one time, but normally the number did not exceed sixteen. Four locusts proved able to start the round-about even without the help of the mill, but without the mill the speeds were always less than those measured in the wind tunnel.

ELIMINATION OF THE DRAG OF THE ROUNDABOUT

In nature a flying locust has only to overcome its own resistance. When suspended in the laboratory, however, it would furthermore have to overcome the resistance of the apparatus (the friction of the ball-bearings and the drag of spokes and suspending bars). This additional resistance decreases the flying speed of a suspended insect compared with the speed it would obtain during free flight. Therefore, in order to make locusts fly at the speed they naturally adopt, a compensation unit consisting of mill and compressor was calibrated in the following way: various numbers of empty suspending bars without terminal plates were attached to the roundabout. The steady speeds of the periphery were measured with a calibrated stroboscope for various settings of the resistance. For any given speed, it was possible to say what setting was required to overcome the resistance of the roundabout for a given number of suspending bars. In an experiment a certain number of locusts were attached to the ring, and at least some of them were stimulated to beat their wings by means of a few flashes of light. The stream of air produced by the flapping wings would stimulate the locust behind to fly. In a few seconds all locusts would beat their wings and start the roundabout. Within a minute the speed would reach about 3 m./sec. even without compensation. But, when the roundabout began to move, the compressor was started and the speed of the ring was measured every 15 sec. At every reading the motor was regulated so as to apply a force just sufficient to overcome the resistance of the roundabout plus the suspending bars at the speed measured. Very soon after the start, i.e. within a minute, the motor could be adjusted to the flying speed, and by continuous regulation of the resistance the locusts could be made to fly at a speed which only depended on the working locusts and the aerodynamic conditions present along the periphery of the roundabout.

In order to measure the resistance of the roundabout, and thus the mechanical advantage of the mill, the middle part of the hub was drum-shaped and functioned as a bobbin on which a thin nylon thread could be wound. One end of the thread was fixed to the bobbin and the other end drawn along the ceiling and passed over a nearly frictionless pulley, the lower end being provided with a pan (Fig. 1). The relation between the loading of the pan and the constant speed imparted to the roundabout was measured. If the loading was F_1, the radius of the bobbin r_1, and the radius of the roundabout r_2, then the force F_2 which should act at the periphery in order to drive the roundabout at the constant speed in question would be $F_2 = \frac{F_1 r_1}{r_2}$.

The relation between the speed of the periphery and F_1 was measured with various numbers of suspending bars, and since r_1 and r_2 were known F_2 could be calculated. In order to measure the parasite drag (= the air resistance of the non-lifting surfaces) of *S. gregaria* eight dead males had their wings cut off and were suspended in the usual way. The relation between the force F_2 and the speed is seen in Fig. 3, which shows the force which should be applied at the periphery in order to drive (1) the empty ring, (2) the ring plus sixteen suspending bars, and (3) eight bars with eight suspended locusts. Curves 2 and 3 are nearly identical. Consequently, the drag of sixteen suspending bars equalled the drag of eight locusts plus eight bars. The drag of one bar therefore equalled the parasite drag of one locust. Let us suppose, for example, that sixteen locusts pulled the roundabout at 3 m./sec. without compensation. The parasite drag of sixteen insects would amount to 3600 − 2600 = 1000 mg. (= 65 mg. per *Schistocerca* ♂). The drag of the wheel and the sixteen suspending bars amounted to 3600 mg. Thus the total drag due to the locusts plus the roundabout equalled 4600 mg. Without the mill the locusts would then have to overcome a parasite drag amounting to 4600 mg. instead of 1000 mg. Of course, they could not do that for long, i.e. they could not manage to fly at 3 m./sec. But with the application of the mill the locusts only had to overcome the natural parasite drag of 1000 mg. It was therefore necessary to use the mill in order to get air speeds of the same order of magnitude as those obtained during natural flight.

SOURCES OF ERROR

In spite of the drag compensation, the flying speed* need not correspond exactly to the air speed which insects, flying in a similar way, would reach in nature because (1) as a rule the locusts did not fold up their hindlegs; (2) the aerodynamic conditions along the periphery might differ from the conditions to which freely flying insects are exposed; and (3) the suspended locusts would not be able to control their lift.

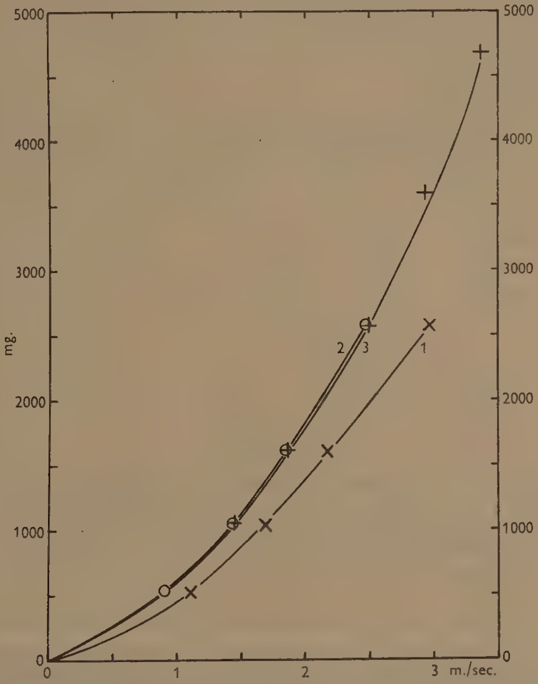

Fig. 3. The force (ordinate) necessary for driving the roundabout at constant speed (abscissa), both the force and the speed being referred to the periphery. 1, the empty ring. 2, the ring + sixteen empty bars. 3, the ring + eight bars with eight suspended locusts. Since 2 and 3 were nearly alike the parasite drag of one locust equalled the drag of one empty suspending bar.

The last objection was considered the most important, whereas the first was regarded of minor significance since the drag of the hind-tibiae could alter the resultant force but little (see later). Therefore only the second and the third objection will be discussed in detail.

The values from the curves in Fig. 3 approximately fitted into the equation $F=kv^x$, where x ranges from 1·6 (curve 1) to 1·7 (curves 2 and 3). The increased resistance at the periphery thus increased the exponent. If the air resistance of the periphery was gradually increased compared with the resistance of the other parts, we should expect $x \to 2$ since air resistance generally increases with the second power of the velocity. The above values correspond fairly well to what might be expected beforehand. It was concluded that the aerodynamic conditions at the periphery did not deviate appreciably from the conditions within migrating swarms in nature, the considerable turbulence of the air being taken into consideration.

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* In order to distinguish the mean speed at which the animals revolve from the well-defined 'air speed' the former was merely denoted 'flying speed'.

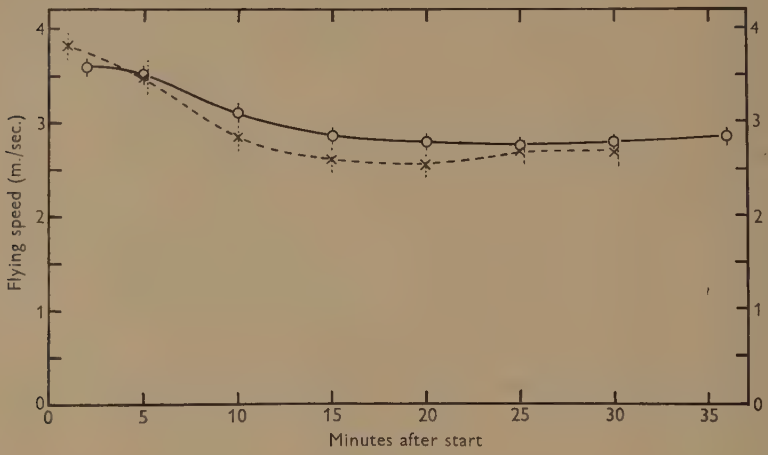

Fig. 4. Comparison between the variations in flying speed (ordinate) during the first half hour of flight (abscissa) of the desert locust when flying in the roundabout (full-line) and in front of a wind tunnel (pecked line) respectively. Each point is the average of several measurements, the standard error of the mean being indicated as vertical lines. The flying speeds, as well as the shape of the curves, are practically identical.

Concerning the failure of the animals to adjust the lift by changing the body angle no direct check has yet been possible, but since the animals were always suspended semi-rigidly and in the same way, all experiments were considered comparable. Moreover, it turned out that the average flying speed as well as the range of speeds recorded in the roundabout were equal to the results obtained with single animals suspended in a pendulum in front of a wind tunnel under the same climatic conditions. This is illustrated in Fig. 4 where the variation in speed during the first 30 min. of flight has been plotted for thirty roundabout experiments and sixteen wind-tunnel experiments respectively. Many other experiments performed under various climatic conditions gave similar results, and since the curves were practically identical it was concluded that the air speeds measured in the roundabout were as valid as the speeds of similarly suspended locusts flying in perfect flight posture in a wind tunnel. By means of an aerodynamic balance such locusts could be shown to be able to lift their own weight.

During the experiment the speed of the periphery, which could be regarded as the mean flying speed of the individuals, was followed continuously by means of a stroboscope (sometimes it was directly recorded on a drum). Thus the speed could be used as a measure of the flight intensity provided all the animals flew. But some animals, here called truants, did not fly continuously apparently for two reasons: first, some locusts very soon learned to place the tarsi on their own wings or to keep the abdomen at bay and get sufficient contact to inhibit flight movements; secondly, some animals would stop flying when exhausted or exposed to unsuitable climatic conditions. In the latter case the degree of truancy is an indicator of the condition of the animals. The truancy due to contact with the wings or the abdomen could be prevented by cutting off the mesotarsi and the metatibiae. This procedure did not impair the animals in any other way provided the wounds were covered with wax. The truancy was measured by observing every fifth minute the number of animals which did not fly and calculated as a percentage of the maximum possible. A truancy of 15 or 20 % had no observable effect on speed, but when the percentage rose above 20 % a reduction of the speed occurred, and at 30 % the experiments were usually stopped. The smallness of the effect of truancy upon the average speed might be due to a low parasite drag of locusts compared with the induced drag, because in wind-tunnel experiments an artificial doubling of the parasite drag of a locust flying at 4 m./sec. caused a decrease in air speed of not more than 0·3–0·5 m./sec. That is why the resistance of the legs was considered less important.

APPLICATIONS AND SOME RESULTS

As a rule the experiments were performed in subdued light regularly interrupted by flashes of bright light, every change in illumination acting as a stimulus. Many other sudden alterations had the same effect, for instance, changes in temperature, sudden noise, etc. Eight to thirty-two locusts could be made to fly continuously for 4–5 hr., the maximum time recorded so far being 9 hr., i.e. flying times comparable with the durations recorded from migrating swarms (Gunn, Perry, Seymour, Telford, Wright & Yeo, 1948). A few results obtained with the desert locust (*S. gregaria* Forsk.) may be anticipated.

(1) Under suitable climatic conditions, i.e. between 25 and 35° C. and a water vapour deficit not exceeding 15 mm. Hg, a 'swarm' of locusts would usually fly for 4–5 hr., and sometimes more, at an average speed of about 3 m./sec., the speed being almost independent of temperature (cf. Weis-Fogh, 1952, table 11).

(2) The initial flying speed was considerably higher, e.g. between 4·0 and 4·5 m./sec., but within half an hour of take-off a rather constant cruising level was reached (Fig. 5 shows a typical experiment). Sudden stimulation resulted in a temporary increase in speed.

(3) In the absence of heating radiation the average flying speed decreased between 25 and 22° C., and sustained flight was never observed below 22° C.

(4) The upper limit for sustained flight was 37° C. and seemed to be raised, rather than lowered, by increasing humidity.

(5) The roundabout has also been used for the study of the metabolism of flying locusts (Weis-Fogh, 1952). A relationship has been established between the

flying speed and the metabolic rate, so that the speed could be used as a measure of the intensity of flight in terms of power.

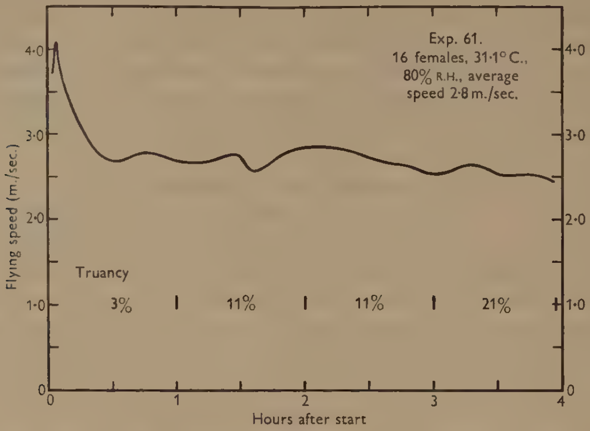

Fig. 5. The variations in flying speed (ordinates) during 4 hr. of uninterrupted flight (abscissa) of sixteen desert locusts, the average speed, the duration of flight, and the uniformity of the performance being typical. The percentages of truancy are given below the curve: up to 15–20 % of truancy did not normally affect the average speed, the parasite drag of locusts being small compared with the induced drag.

SUMMARY

A roundabout technique is described which makes it possible to study the flight performance of a small 'swarm' of locusts (up to thirty-two individuals) for hours at a time. The resistance of the roundabout was compensated by means of a mill so that the locusts only had to overcome their own air resistance. The speed of the revolving periphery therefore equalled the preferred average flying speed of the suspended locusts.

The average speed during a period, as well as the variation in speed in the course of an experiment, were found to be the same in the roundabout and in experiments where single locusts flew in front of a wind tunnel. In the latter case the insects flew in completely normal flight posture. It was concluded that the results obtained with the roundabout were as valid as the results obtained with a wind tunnel.

Some preliminary results are given on the influence of different external factors on the flying speed and the ability to endure sustained flight.

We are indebted to the Anti-Locust Research Centre, London, the Carlsberg Foundation (Carlsbergfondet), and to the Scandinavian Insulin Foundation (Nordisk Insulinfond) for financial support. Thanks are due to Dr D. L. Gunn and to Dr C. O. Nielsen for suggestions and linguistic improvements. We furthermore thank Mr Helge Nielsen, engineer, for technical assistance in the construction of the roundabout.

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THE RESPONSE OF A SENSE ORGAN TO A HARMONIC STIMULUS

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(With Plate 9 and Eight Text-figures)

INTRODUCTION

Since the classical researches of Adrian (1926), in which electrical amplification techniques were first used to detect the pattern of impulses which pass up a sensory nerve when its end-organ is stimulated, a large number of different types of receptor have been studied by this method. Its attraction is that it gives direct information about the quality of stimulus to which the ending responds and also, when the activity of a single nerve fibre can be observed, provides quantitative measurements of the relationship between the magnitude of the stimulus and the frequency of the nervous response. From such experiments have come the most important generalizations about the properties of receptors (Adrian, 1928). The electrical recording technique in nerves and nervous tracts is now the most useful method available to the physiologist not only for the investigation of sense organs but also in the analysis of the working of the rest of the nervous system.

From another point of view, however, this powerful technique has so far proved disappointing. Detailed analysis of the properties of sense organs, nerves and muscles has not led to an increase in the accuracy of our knowledge of the physiological composition of the patterns of movement which make up the behaviour of the intact animal. The work done by Sherrington and his co-workers on the reflex activity of the spinal vertebrate remains the basis for subsequent investigations of animal movement at this level of analysis. The concepts introduced by him have provided the framework for analytical researches such as those of Gray & Lissmann (1946) on the toad, and of Pringle (1940) on Arthropods. Workers in this field have been concerned to demonstrate qualitatively the existence of reflexes or inherent movement patterns and to show how these are related to the normal behaviour of the intact animal. In few cases has the reflex or basic movement pattern been given quantitative definition, and in none has the attempt been made to analyse further this quantitative description in terms of the properties of the sense organs, nerves, muscles and portions of the central nervous system which combine to produce the observed activities of the preparation.

The difficulties of such a further analysis are considerable. Not the least is the lack of detailed knowledge of the anatomy of the nervous system of most of the animals which have been studied, and the great numerical complexity of the cells whose co-ordinated functioning produces the response observed. The Arthropods have the advantage over the Vertebrates in this respect; the work on Crustacea (Wiersma, 1941) and insects (Pringle, 1939) has shown that, at any rate on the motor side of the reflex arc, the number of nerve cells which have to be considered may be conveniently small. But lacking also has been the theoretical basis for the synthesis of an understanding of the functioning of the whole of a complicated dynamic system from a knowledge of the functioning of each of its parts. Help has recently come to biology in this matter from an unexpected source. The efforts of engineers to design satisfactory and stable automatic control machinery have led to the development of a comprehensive theory of such self-regulating devices or servomechanisms, and it has come to be recognized that the long-established self-regulatory properties of living organisms (Cannon, 1939; Holmes, 1948 and earlier papers) imply a similarity in mode of functioning between organisms and some of these man-made machines. The similarity resides ultimately in the mathematical expressions adequate to describe the functioning of the two types of system. While there is not yet agreement among biologists about the value of some of the analogies which may be drawn between the functioning of the living and the non-living, there can be no doubt that much may be learned from the methods used by control-system engineers in the analysis and synthesis of self-regulating machinery. The neurophysiologist, who is still at the stage of preliminary analysis, can be grateful for any hints that may enable him to plan his experiments economically, and to acquire his results in a form that will make easier the ultimate task of synthesis of an understanding of the working of the whole of the nervous system and, ideally, of the whole of the living body.

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The principles of the Theory of Control are now well described in a number of text-books, such as that of Macmillan (1951). The methods used in the analysis of a complicated piece of automatic machinery repay a short examination in order to see how they are related to the methods of the physiologist in his investigations of the living organism. The engineer has available to him several points in the control circuit at which, with the aid of instruments, he can measure the state of the machinery. He can also apply controlled 'disturbances' which alter the state of the machinery in a manner which can be measured. He may be obliged for various reasons to make his measurements without disturbing the natural running of the machine. On the other hand, it may be possible for him to stop its normal operation, or the operation of portions of it, in order to study the properties of the components in partial or complete isolation. If the machinery is fully automatic it will involve, in some part of its mechanism, a 'closed-loop' connexion between its constituent parts; that is, a part A whose operation is *controlled by* another part B will also, through a greater or smaller number of intermediate stages, *control* part B. This is a universal feature of all fully automatic or self-regulatory systems. More than one such closed-loop may be present in the machinery, and the engineer may be able to 'open' one or more of these by disconnecting one part from another in order to make his measurements.

These analytical procedures have their exact counterparts in the methods of the physiologist—the preliminary observation using techniques which do not affect the animal as a whole; the application of 'stimuli' producing 'responses' of various kinds; the interruption of normal working by anaesthetics: and the disconnexion of parts by dissection. Were it not for the confusion caused by the growth of two different terminologies to describe the same procedures, the similarity would no doubt be more widely appreciated.

One of the most obvious ways in which the technique of the biologist is inferior to that of the engineer is in the nature of the disturbances or stimuli used. The engineer, with the goal of mathematization always before him, uses disturbances whose time course is planned to facilitate subsequent mathematical manipulation of the results. To the biologist a stimulus often means merely a quantitatively undefined change in the intensity of some quality in the environmental situation. When it is more exactly specified, as in physiological experiments, the definition usually covers only the *magnitude* of the change and does not concern itself with the *rate of change*. The change may be made as rapidly as experimental conditions allow, and where the rate of response is slow this approximation to a sudden 'transient' is adequate for the purpose. But it is a type of disturbance which leads to results in a form which is not ideal for all purposes, and the form is particularly unsuitable when the objective is a synthesis of an understanding of the dynamic operation of the whole of the mechanism or the behaviour of the animal as a whole.

For such a synthesis it is necessary that the properties of each of the component parts of a control circuit be expressed in a form which makes possible the combination of the results into a statement or representation of the properties of the whole circuit. With this in mind, engineers have developed the concept of the 'transfer function', which may be either a mathematical expression or a graphical representation of such an expression, and with the aid of which it is possible to describe accurately and fully the response which is given by the component when a disturbance of any known time course is applied to it. A mathematical expression naturally carries with it an assumption of accuracy which may be unjustified in biological work, and graphical forms may be more suitable in this context; but with either the procedure is well worked out for the synthesis of an understanding of the properties of the whole from a knowledge of the properties of the parts once these are expressed in suitable form. A note of the different forms of transfer function is included as an appendix to this paper.

The task of synthesis is made easier for the neurophysiologist by one fortunate circumstance. Transfer functions in mathematical or graphical form may be multiplied together to give the transfer function for the whole control sequence only when the component parts are non-interacting. The all-or-none nature of the transmission of excitation in nerve fibres and the irreversibility of synaptic connexions between fibres establish this non-interaction condition in many parts of the nervous system, and, provided that due allowance is made for the finite delay involved in nervous transmission, there is a sound theoretical basis for a reconstruction of the functioning of a complete reflex arc from a knowledge of the properties of the sense organs, nerves, central structures and neuromuscular systems concerned. It is with this possibility in mind that the present investigations have been undertaken.

As will be clear from a perusal of the procedures summarized in the Appendix, the derivation of the transfer function of a component from results obtained by the use of transient disturbances is laborious and carries the danger of serious errors. For linear systems (a linear system is defined as one in which the magnitude of the response is directly related to the magnitude of the stimulus) the use of harmonic (sinusoidal) disturbances greatly simplifies the subsequent calculations. With this type of stimulus the response is always also sinusoidal; the properties of the component can therefore be described in terms of three parameters, a simple *proportionality* of magnitude and a *phase* relationship between input and output for each *frequency* of harmonic stimulation. A single vector diagram thus contains a full statement of the results in a form which may be used directly for subsequent synthesis procedures. Although the assumption of linearity is often unjustified in biological systems when a wide range of stimulus intensities has to be considered, it can serve to initiate an understanding of the behaviour of the system and to indicate the nature of the effects produced by departures from linearity. Transfer functions for systems containing non-linear elements apply adequately to a particular amplitude of stimulus or input disturbance, and provided this limitation is borne in mind they may be used to build up a picture of the functioning of the whole.

An investigation of the properties of a sense organ from this point of view is described in this paper, the object being not to reveal any essentially new feature of sensory neurophysiology, but to re-describe the well-known phenomenon of sensory adaptation in a manner which may be of more direct value in an understanding of the functioning of the whole response mechanism of which the sense organ is the initial part. The work was undertaken more in order to test the possibilities of the method than with the intention of pursuing the investigation to a high degree of accuracy. For this reason a sense organ was chosen which is easy to study in isolation and from which single nerve-fibre oscillograph records can be obtained with a minimum of dissection.

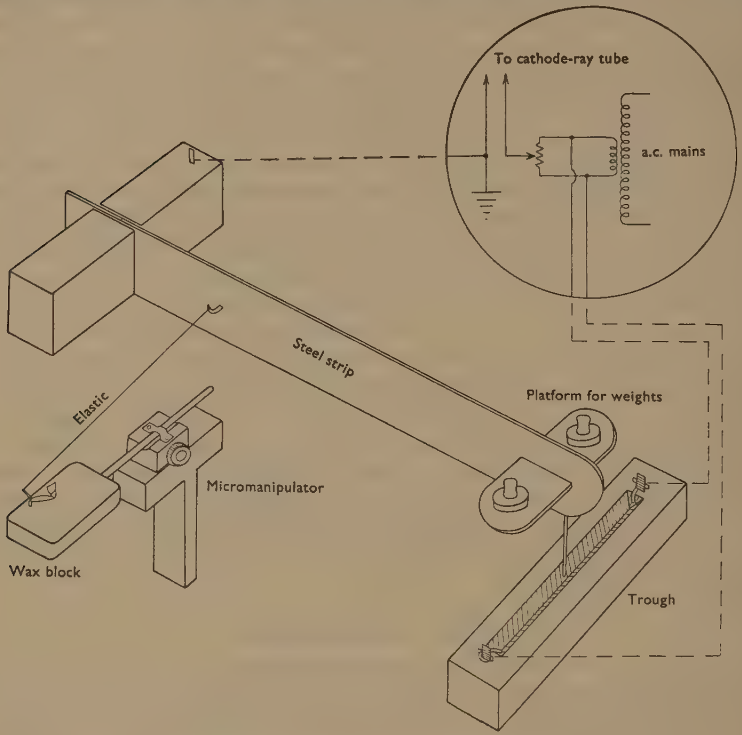
MATERIAL AND METHODS

The thoracic legs of the American cockroach, *Periplaneta americana* L., bear on the tibia and femur a series of large spines each of which is innervated by a single sensory nerve fibre of large diameter. The response of this ending to mechanical stimulation was described by Pumphrey (1936), who drew attention to the fact that the rate of adaptation to a constant deflexion of the spine is slow compared with that of the tactile receptors described by Adrian & Zotterman (1926). After some preliminary experiments with various tibial spines, a choice was made of the single large spine on the dorsal surface of the end of the femur of the metathoracic leg, which gave consistent results in many individuals.

Impulses in the sensory fibre supplying this spine may be recorded with platinum wire electrodes inserted through the cuticle at opposite ends of the femur without any dissection, and the isolated femur preparation survives for sufficiently long for experiments to be performed. The simplicity with which single nerve-fibre records may be obtained with this preparation is a great advantage.

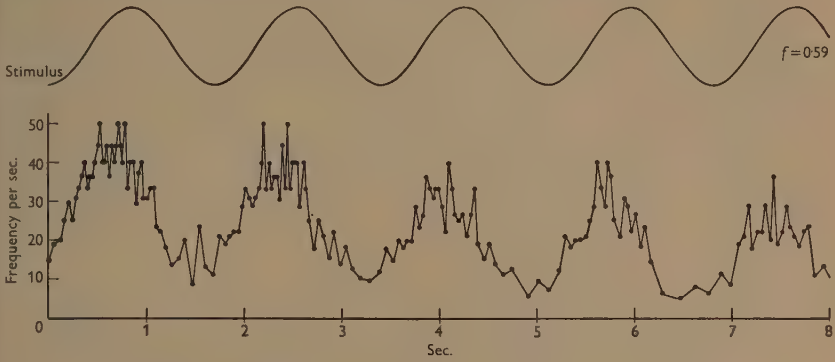
The electrical amplifying and recording system is standard. A Grass P4 pre-amplifier was used together with the built-in amplifiers of the Cossor 339A oscilloscope.

The generation of a sinusoidally varying mechanical stimulus of variable frequency was achieved by means of a steel strip, clamped at one end and at the other carrying a heavy weight, which thus oscillated in a horizontal plane (Text-fig. 1). A copper wire from this strip dipped in a trough filled with a saturated solution of copper sulphate which formed the variable branch of an a.c. bridge circuit, the unbalance current from the bridge being fed to the second plate of the oscilloscope to give an indication of the amplitude and phase of the mechanical stimulus. To avoid the frequency doubling inherent in this type of indicating circuit the bridge was purposely unbalanced at the mean position of the oscillating strip. At the stimulation frequencies required a satisfactory indication of the stimulus was given by feeding the bridge through a step-down transformer from the a.c. mains (50 cycles/sec.).



Text-fig. 1. Diagram of experimental apparatus.

To transmit the stimulus to the femoral spine, connexion was made between its tip and the steel strip by a short length of fine elastic, and the preparation on its wax block was then adjusted by means of a micromanipulator so that the elastic was just taut at the end of the swing of the oscillating strip. The leg spines of the cockroach have a small freedom of movement in their sockets and this movement produces slight excitation of the sensory ending, but the more effective and natural method of excitation when it is pressing against an object on the ground is with the spine against its limiting stop in the socket. In this position (which was used in the experiments) its movement is negligible and the adequate stimulus is a change of tension. The sinusoidal changes of stimulus intensity were thus transmitted to the spine without distortion. The peak tensions applied in this way were about 5 g.



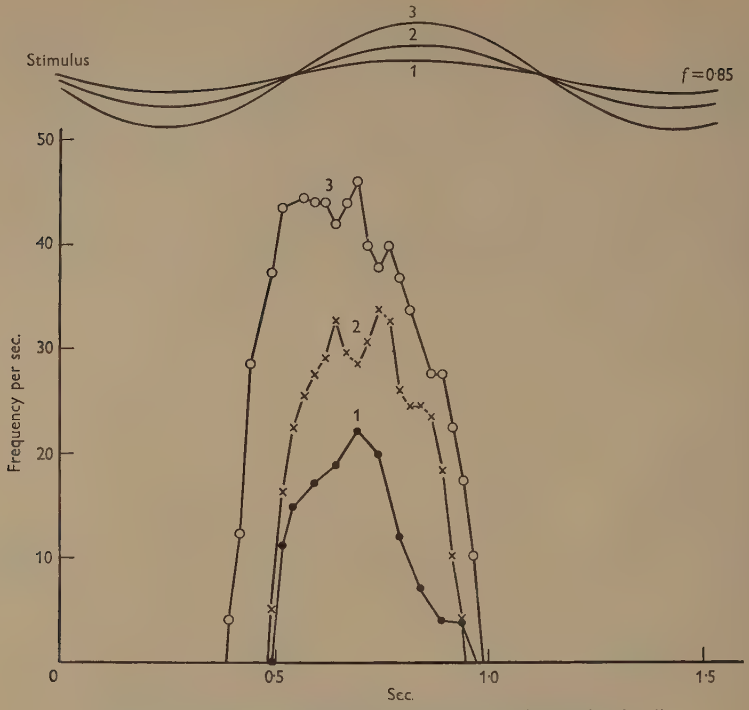
Text-fig. 2. Response of the femoral spine ending to a low-frequency harmonic stimulus superimposed on a steady tension.

METHOD OF ANALYSIS

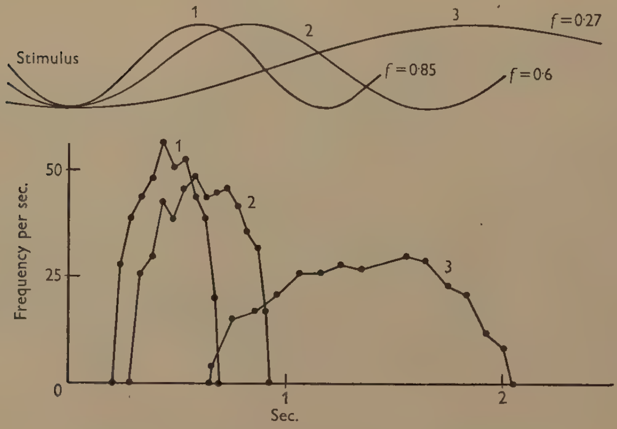
Typical oscillograph records are shown in Pl. 9.

The analysis of these records is carried out as follows. A zero point in the cycle of stimulation is first chosen, conveniently at the narrowest part of the stimulus trace (minimum tension on the spine). By measurement from this point a graph is plotted showing the relationship between instantaneous impulse frequency (the reciprocal of the interval between one impulse and the next) and the instant of time to which this refers. In Text-figs. 2–4 each frequency measurement is referred to the instant of time half-way between the two impulses.

Except in Text-fig. 2, where the frequency graph has been plotted over five consecutive cycles of stimulation, the graphs for successive cycles are superimposed and an average graph calculated in order to reduce the random scatter. The graphs of Text-figs. 3 and 4 are obtained in this way.



Text-fig. 3. Response of the femoral spine ending to harmonic stimuli of different amplitudes.



Text-fig. 4. Response of the femoral spine ending to harmonic stimuli of different frequencies.

RESULTS

The records of Pl. 9 show, as may be expected, that the sensory ending is excited only when the tension on the spine reaches a threshold value. To avoid this effect, which makes the frequency graphs discontinuous, the harmonic stimulus was sometimes superimposed on a steady tension sufficiently great to prevent the frequency of impulses falling to zero. The record from which Text-fig. 2 is plotted was obtained with this arrangement; the frequency graph then becomes sinusoidal with superimposed random fluctuations.

It will be noted that the maximum response regularly precedes the maximum of the stimulus. Experiments were carried out to determine how the amplitude and phase-advance angle varied: (1) with change in the amplitude of the stimulus; (2) with change in the frequency of the stimulus.

Text-fig. 3 shows a plot of an experiment in which the tension was allowed to fall just below threshold at the troughs of the stimulus cycle and in which the frequency plot is therefore discontinuous. The stimulus amplitude was varied at constant frequency. The peak response occurred at a point in the cycle corresponding to a phase advance of approximately 45° and showed a tendency to occur earlier as the amplitude of the stimulus was increased. The amplitude relationship also shows some non-linearity.

Text-fig. 4 shows a plot of the records of an experiment in which the frequency of stimulation was varied at constant amplitude. The phase advance angle of the maximum response for the three frequencies of stimulation (0·85, 0·6, and 0·27 cycles/sec.) is, respectively, 45, 51 and 49°, and the amplitudes of the peaks of the response are in the ratio 54 : 47 : 29. These results are plotted on the graphs of Text-figs. 7 and 8.

DISCUSSION

The results obtained do not cover a sufficiently wide range of stimulus frequencies and amplitudes to permit an accurate evaluation of the transfer function for this sense organ, nor to show quantitatively how this changes with stimulus amplitude owing to the non-linearity of the ending. They do, however, show that with a slow harmonic stimulus the ending produces a phase advance in the plot of nerve impulse frequency against that of the stimulus. Owing to the presence of a random scatter in the timing of individual impulses the estimates of phase advance can only be made approximately, and for the same reason frequencies can only be used which are sufficiently slow to produce a considerable number of impulses at each cycle of stimulation. At higher frequencies, which ultimately produce only one or two impulses per cycle, it would be necessary to adopt a different measure of nerve activity to indicate quantitatively the excitation of the ending.

The phase-advancing property which has been demonstrated is a corollary of the adaptation shown by experiments on sense organs using transient stimuli, since adaptation implies a response to the rate of change or time derivative of the stimulus as well as to its instantaneous value. The connexion between the derivative response to transient stimulation and the phase advance using harmonic stimulation is discussed in the Appendix.

The non-linearity of the response of the sense organ is shown in Text-fig. 3, the phase advance increasing and the proportionality of magnitude decreasing as the stimulus is increased. Evidence from other types of mechanical sensory ending (Matthews, 1933; Fessard & Sand, 1937), in which the frequency of nerve impulses has been shown to be approximately proportional to the logarithm of the stimulus intensity, also suggest that this element in the reflex arc cannot be regarded as a linear component.

The existence of the phase-advancing property in sense organs has a number of interesting implications when their role in the intact animal is considered. Reflex arcs whose function is to regulate the level of some aspect of vital activity are known to occur in many animals. The best known of them is perhaps the myotatic reflex of the vertebrate, in which the stretch produced in muscle sense organs by an increase in tension on the muscle as a whole leads to an increase in the tone of the muscle (Creed, Denny-Brown, Eccles, Liddell & Sherrington, 1932). The control sequence in this reflex includes the delay involved in nervous conduction to and from the central nervous system, and the central and neuromuscular delays. The theoretical analysis of automatic control systems shows clearly that a regulating system of this sort with finite delays in the control sequence is unstable and gives rise to oscillations or 'hunting' if some form of compensation is not included. The performance is made worse if there is any inertia in the load against which the muscle is working. The introduction of a phase advance in one of the components in the control sequence provides the required compensation, provided that the ratio of derivative to direct response of the component is greater than the total delay time and that the overall gain of the system (e.g. in this case the muscle tension produced reflexly by a given stimulus tension) is not too great (Macmillan, 1951). This function of sensory adaptation has been recognized by Merton (1951), who has demonstrated its quantitative significance in the intact reflex in man by studies of the timing of the silent period in the motor discharge which occurs when a stimulus in the form of an increase in tension is applied to the muscle.

Another reflex in which the regulatory properties of a closed control circuit is of importance is the Hering-Breuer reflex of mammals, by which the depth of respiratory movements is regulated through the action of strength receptors in the lungs and bronchi. The action of this reflex is to produce not a steady maintained state as in the myotatic reflex in postural muscles but a rhythmic oscillation of inspiratory and expiratory movements. It has been demonstrated that the integrity of the vagus nerves carrying the afferent fibres for this reflex is, in the low decerebrate preparation in the cat, necessary for the continuation of these rhythmic movements (Pitts, 1942). It is perhaps significant that the lung stretch receptors appear to show a much smaller derivative component in their response to transient stimuli than do the stretch receptors in postural muscles (Adrian, 1933; Matthews, 1933). Without a knowledge of the nerve conduction times and central delays involved in the Hering-Breuer reflex, it is not possible to make an estimate of the stability to be expected from this particular control mechanism, but a reduction of the derivative response in the sense organs certainly favours the generation of rhythmic oscillations by such a mechanism.

Harmonic stimulation of mechanical receptors is most nearly approached under natural conditions in the swimming movements of fish. If reflex arcs similar in function to the myotatic reflex of mammalian limbs are present in the trunk neuromuscular system of fishes (see Gray, 1936), the relation of these reflexes to the inherent rhythm of activity of the spinal cord (i.e. whether they will reinforce or antagonize the inherent rhythm) will be determined by the phase relationships round the reflex arc, and to these the properties of the sense organs will make a significant contribution.

In any analysis of the phase relationships round a complete reflex arc the integrative properties of junctions have also to be considered. The liberation of a chemical mediator of synaptic or neuromuscular transmission whose concentration is related to the frequency of arriving impulses implies a phase lag in this part of the control sequence, and the rates of production and destruction of such a mediator provide another variable which will affect the overall performance of the reflex arc. The slow potential changes observed by Barron & Matthews (1938) to accompany the initiation of impulses at the spinal origin of motor fibres also provide an intermediate point at which the level of excitation may be measured, and the evidence that the frequency of impulses here as in a sense organ is related both to the magnitude of the preceding event and to its rate of change suggests that the derivative response or phase-advancing property is present in this type of excitable nerve cell as well as in the sensory fibres.

The picture presented by this type of analysis of the nervous system is of a series of cell elements affecting each other in a complex manner which is nevertheless capable of quantitative description in terms of a suitable transfer function whenever the all-or-none impulse transmission connexions can be reached by the exploring electrode of the neurophysiologist. In terms of the harmonic analysis here outlined, each synaptic connexion will produce for each frequency a certain magnitude relationship and a certain phase relationship when the input to and output from the synapse are measured as the frequency of nerve impulses. A new light is thrown on certain purely central phenomena by this analytical approach. In the more complicated centres of the central nervous system it has long been assumed (see Lorente de No, 1938) that some at least of the neural connexions involve closed loops of neurons, part of whose function is the maintenance of activity in the absence of peripheral stimulation. The analysis of neural interaction in terms of magnitude and phase relationships provides a new picture of the way in which activity may be maintained by such closed connexions, since an oscillatory condition will be produced in any such loop in which the total phase change equals a multiple of 360° and the total magnitude relationship is unity. The frequency of the resulting oscillation will be such as to preserve this total phase change and its amplitude will be determined by the non-linearities of the component elements. This explanation of the origin of, for example, the cortical electrical rhythms of the mammalian brain is in some ways preferable to the original concept of 'reverberatory arc' in which the individual impulse is supposed to preserve its identity in its passage through the synaptic maze. It postulates that the smooth potential wave-forms which can be recorded from the cortex are the sum, not of a large number of impulse spikes, but of a number of synchronized potential changes in the neurons each of which has an approximately sinusoidal time-course, and each similar in nature to the slow potential waves which were observed by Barron & Matthews (1938) in the dorsal and ventral horns of the spinal cord.

The analysis clearly becomes inapplicable in the case of junctions where there is appreciable synchronization between presynaptic and postsynaptic impulses, just as it fails in the case of sense organs at stimulus frequencies which produce only a few impulses per cycle. But it is not clear that such synchronization across synapses is as common under normal conditions as it is when the presynaptic volley is produced artificially by maximal stimulation of the sensory nerve trunk. Detailed neurophysiological examination of reflex connexions between single nerve fibres in the central nervous system has as yet been reported only in the case of giant fibres (Wiersma & Turner, 1950), whose synapses are not concerned with maintained activity in the muscles.

APPENDIX

The form and derivation of the transfer function

The transfer function for an element in a control system is ideally defined in the form of a mathematical expression with the aid of which differential equations may be derived for the behaviour of the element for any type of input disturbance. Procedures are available for the derivation of transfer functions from the results of experiments using either transient or harmonic (sinusoidal) inputs, but the computation involved in the use of results of the latter type is much less cumbersome.

Transfer functions in mathematical form are commonly expressed in one of three ways:

(1) With the Heaviside differential operator $D\,(=d/dt)$.

(2) In terms of the parameter p (sometimes s) defined by the Laplace transformation

$$\mathscr{L}\{F(t)\}=f(p)=\int_{0-}^{\infty} e^{-pt}F(t)\,dt.$$

(3) In terms of complex numbers in the form $M(j\omega)$.

Graphical representations of transfer functions are based on the Argand diagram, using the identity

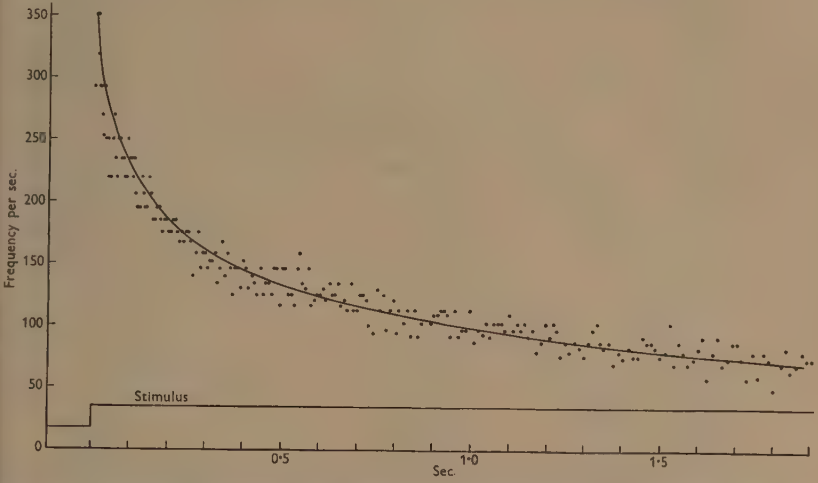
$$M(j\omega)\equiv Ae^{-j\psi}.$$

When the proportionality of magnitude (A) and the phase angle (ψ) between input and output are known from observations over a range of input frequencies, a vector diagram in the complex plane may be plotted direct in polar co-ordinates and used in synthesis procedures without any attempt to remove experimental error by the fitting of mathematical formulae to the observed measurements.

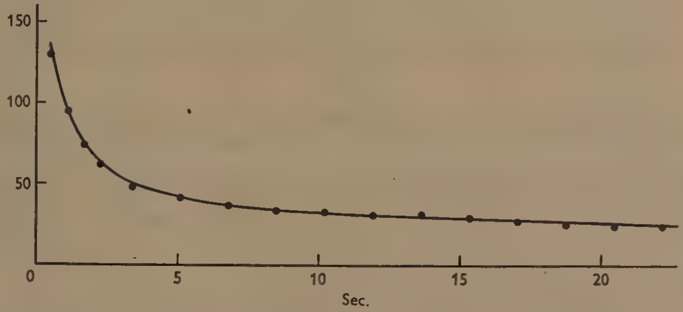
To illustrate these procedures an example is now worked out in the different forms. Text-figs. 5 and 6 show the response of the femoral spine sensory ending to a transient stimulus produced by suddenly increasing the tension on the spine by means of a rapidly acting electric relay. To the points which represent the instantaneous frequency of impulses in the sensory nerve (plotted as previously described) has been fitted a smooth curve of the equation

$$\text{frequency} = 24 + 165e^{-12t} + 120e^{-1\cdot1t} + 42e^{-0\cdot175t}. \tag{1}$$

The fit is good within the limits of natural scatter of the observations.



Text-fig. 5. Response of the femoral spine ending to a transient stimulus. The points are plotted from measurement of the oscillograph record and the curve is that of Equation (1).

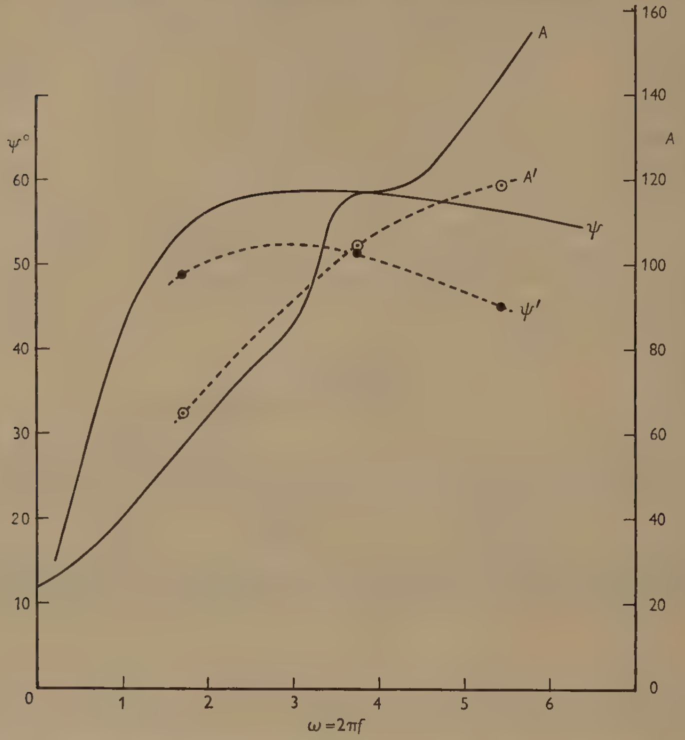


Text-fig. 6. Continuation on a longer scale of the record plotted in Text-fig. 5.

The transfer function derived from this equation by the use of the Laplace transformation is

$$f(p) = \frac{351p^3 + 789p^2 + 486p + 57}{p^3 + 13\cdot3p^2 + 15\cdot5p + 2\cdot3} \tag{2}$$

with initial conditions of zero activity in the nerve. Under such conditions the Heaviside differential operator D can replace p to give the transfer function in form (1) above. The response of this sense organ thus appears to involve the second and third derivative of the stimulus as well as the first.



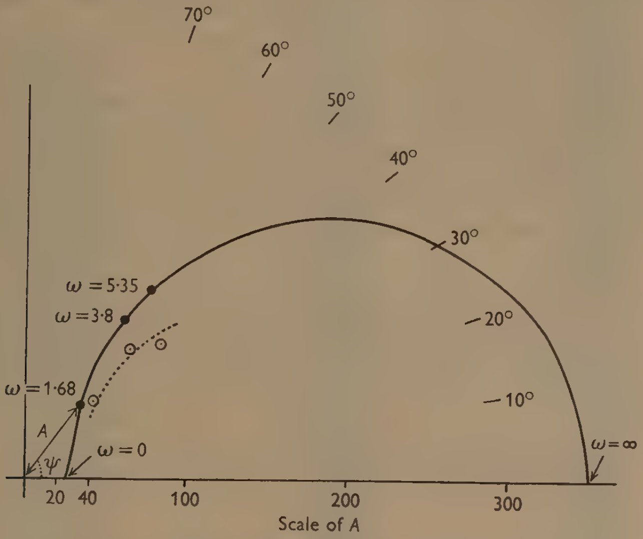
Text-fig. 7. Observed (dotted) and calculated (continuous) curves for magnitude proportionality A and phase angle ψ for the response of the femoral spine ending to a harmonic stimulus.

To derive the harmonic response locus from the transfer function in the form of expression (2) we may substitute $j\omega$ for p and express the result in the form $Ae^{-j\psi}$. This gives

$$M(j\omega)=\frac{\sqrt{[(57-789\omega^2)^2+\omega^2\,(486-351\omega^2)^2]}}{\sqrt{[(2{\cdot}3-13{\cdot}3\omega^2)^2+\omega^2\,(15{\cdot}5-\omega^2)^2]}}$$

$$\times\exp\left[\tan^{-1}\left\{\frac{\omega\,(486-351\omega^2)}{57-789\omega^2}\right\}-\tan^{-1}\left\{\frac{\omega(15{\cdot}5-\omega^2)}{2{\cdot}3-13{\cdot}3\omega^2}\right\}\right].$$

The calculated magnitude proportionality A and the phase advance angle ψ are plotted separately on Cartesian co-ordinates in Text-fig. 7 and together as a vector diagram in Text-fig. 8, for the range of frequencies from 0 to ∞. The observed results from the experiment of Text-fig. 4 are also shown on these graphs, to indicate the correlation between the results obtained with the two types of input stimulus. It should be borne in mind that the experiments were done with preparations from two different individuals.



Text-fig. 8. Vector plot of the observed (dotted) and calculated (continuous) response of the femoral spine ending to a harmonic stimulus.

SUMMARY

1. The response is described of the tactile ending in a femoral spine of the leg of *Periplaneta* to a harmonic (sinusoidal) mechanical stimulus of low frequency. The peak frequency of impulses in the sensory nerve precedes the maximum tension of the stimulus.

2. This result is shown to be a corollary of the adaptation shown by the sensory response to a transient stimulus.

3. The concept of the 'transfer function' is discussed in relation to neurophysiology. Its value is explained as a means of describing the dynamic properties of component parts of the nervous system when the objective is an understanding of the functioning of the complete reflex arc.

The continued interest of Mr R. H. Macmillan has been of great assistance in this work.

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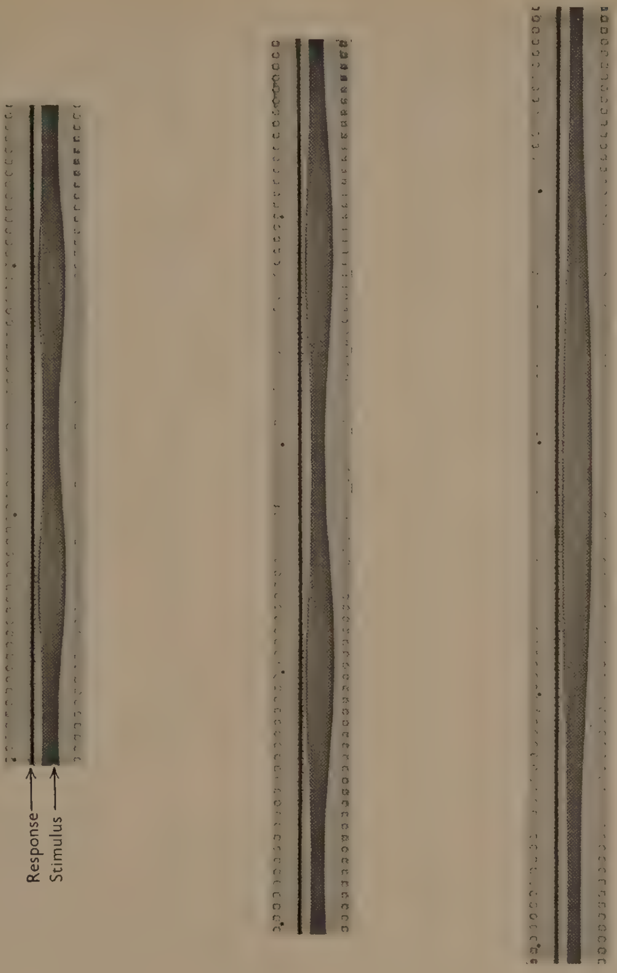
EXPLANATION OF PLATE 9

Response of the nerve ending in the femoral spine of *Periplaneta* to harmonic stimulation a frequencies of 0·85, 0·6 and 0·27 per sec. Time marker 1/sec.



PRINGLE AND WILSON—THE RESPONSE OF A SENSE ORGAN TO A HARMONIC STIMULUS

FACILITATION IN SEA ANEMONES.

III. QUICK RESPONSES TO SINGLE STIMULI IN *METRIDIUM SENILE*

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(*Received* 3 *October* 1951)

(With Ten Text-figures)

INTRODUCTION

Quick protective closing movements are a feature of the behaviour of the sea anemones *Metridium senile* and *Calliactis parasitica*. By studying the way in which these movements can be elicited by electrical stimulation, Pantin (1935 *a–d*) has shown that the response is controlled by a process of facilitation, developed here to a unique degree.

A single electrical stimulus applied anywhere on the column of the animal completely fails to bring about a quick response. A series of stimuli at intervals of about 3 sec. or longer is similarly ineffective, though such a series may cause slow movements of various kinds after some delay. If the interval between stimuli is shortened, a point is reached where tiny quick-closing movements occur to every stimulus after the first. Further shortening of the interval has the effect of increasing the size of the separate quick-closing movements which occur on the second and subsequent stimuli. Apparently the only effect of a single electrical stimulus is to set up a state of facilitation which falls off over a period of 2 or 3 sec. A subsequent stimulus is only effective if it is given before the state of facilitation has decayed, and the size of the response to such a stimulus will depend on how far the decay of the facilitated state has gone.

Published accounts of the nervous system and muscles of actinians do not throw much light on where and how this process of facilitation takes place. However, by showing that the ineffective stimulus throws the whole nerve net into the refractory state, Pantin (1935 *a*) established that the facilitation process occurs at or near the neuro-muscular junctions. He therefore called it neuro-muscular facilitation.

Some of the physiological properties of neuro-muscular facilitation emerged from investigations on the influence of various treatments upon it. Hall & Pantin (1937) showed that the process was affected by temperature changes; the facilitated state could be prolonged by cooling and shortened by warming the sea water bathing the anemone. Subsequent papers described the changes in the quick response induced by various ionic conditions and drug treatments (Ross & Pantin, 1940; Ross, 1945 *a*). In general, the character of the response was not altered by any of these treatments. Excess K^+ and Ca^{++} increased, and excess Mg^{++} diminished, the size of the response at any given frequency, but without altering either the duration of the facilitated state, or the rule that every stimulus except the first is effective within the appropriate frequency range.

Most of the drug effects followed the same pattern. Some drugs, especially tyramine, 933 F and cocaine, increased, others, including ergotoxine and trimethylamine, decreased the size of the response, but again without affecting the duration of facilitation. Yet some of these treatments did occasionally alter the rule that only stimuli after the first were effective. After tyramine and 933 F particularly, and with several other biological amines to a lesser extent, *Calliactis* showed a tendency to respond to single shocks during certain phases of the treatment. This evidence that a few substances having effects at adrenergic nerve-endings elsewhere had facilitating effects on the anemones was consistent with the view that facilitation was a neuro-humoural process carried out by a 'facilitator'. Attempts were made, therefore, to see whether substances with facilitating properties, i.e. inducing responses to single shocks and prolonging the facilitated state, could be detected in material extracted from the anemones themselves. Preliminary results of such tests have been described (Ross, 1945 *b*), and these showed that responses to single stimuli did occur frequently following treatment with extracts of both *Calliactis* and *Metridium*. These responses to single stimuli, unlike those with such drugs as tyramine, occurred very soon after the extract was introduced (1–5 min.). Furthermore, they were generally not accompanied by changes in the size of the response, and the rate of decay of facilitation was not affected. Thus, notwithstanding the responses to single stimuli obtained by this treatment, it could not be maintained that the active material in the extract possessed all the properties that would be expected of a 'facilitator'.

This paper deals with further tests undertaken to clarify the situation in which the earlier work had left the problem. In the main, the experimental work consists of a new description of the effects of anemone extract on *Metridium*. This description includes data on the frequency of the occurrence of responses to single stimuli, on the potency of various extracts and on temporal aspects of the effects. But it was found early in the work that responses to single stimuli occurred with certain other treatments, and so a survey of the effects of a variety of substances and conditions forms part of the account presented here. It might be as well to state in advance that the work has led to the exclusion of the possibility that the extract effects are due to the activity of a 'facilitator'. In spite of this, the effects are still important because from their nature it is possible to learn a good deal about the quick response and the processes of excitation which initiate it in the normal animal.

METHODS

The effects of the extracts were studied in essentially the same way as in the earlier work. Minced anemones, usually five or ten at a time, were extracted with cold ethyl alcohol, and the extract so obtained was concentrated under reduced pressure to about one-twentieth of its original volume. Tests were carried out by introducing small quantities of this concentrate (diluted in sea water) directly into the sea water bathing the test animal.

The effects of the extract on a sea anemone were observed in the earlier work by giving single stimuli at irregular intervals from a stimulator delivering condenser discharges through fluid electrodes. In the work now reported it seemed desirable, using the same stimulator, to send in shocks at regular intervals well outside the frequency at which quick responses normally occur. The frequency most commonly employed was one stimulus every 10 sec. It was expected that such experiments would show when and how often the responses to single stimuli occur, and how long the effect lasts. Moreover, the use of a standard repeatable procedure should allow quantitative comparisons of the potency of different extracts and treatments to be made.

Some of the tests were recorded on smoked paper, and these give a continuous graphical record of the results. But even very tiny quick responses are easily detected by eye, so where data on the number of responses and the times at which they occurred were all that was required, it was the usual practice to follow the experiment visually and record these data for controls and treatments alike.

QUICK RESPONSES TO SINGLE STIMULI WITH *METRIDIUM* EXTRACTS

Some qualitative features

Preliminary experiments confirmed the earlier observation that following the introduction of small quantities of *Metridium* extract into the sea water, *Metridium* frequently responds to single stimuli. These preliminary tests showed that if this effect occurred at all, it could usually be detected within the first 3 min. of the treatment. It therefore became the standard practice to continue the tests only for 3 min. after the introduction of the sample of extract.

Figs. 1 and 2 show two typical smoked records taken during treatments of *Metridium* with *Metridium* extract and bring out clearly the character of the effects induced. The control series shows the typical absence of quick responses in an untreated animal stimulated at a frequency of one shock every 10 sec. and the response to a pair of shocks 1 sec. apart at the end. Following the introduction of the extract, responses occur occasionally to isolated stimuli in the series, to shocks at 10 and 20 sec. in one example and 1 min. 50 sec. in the other. Clearly the effect of the extract is to pave the way for quick responses only to occasional stimuli, not to every stimulus over an extended period. This sporadic aspect of the effect was not so evident in the earlier work, and its significance will need to be carefully considered. Fig. 3 shows a similar result with stimuli every 15 sec. in which quick responses occurred 15 sec. and 2 min. 15 sec. after the beginning of the treatment. Fig. 4 shows the same type of record with stimuli every 5 sec. At this frequency slow contractions occur which are reflected in the lowering of the lever after five stimuli in the control series. Following the treatment quick contractions occur at 15 sec. and 1 min. 55 sec., then to three successive stimuli beginning at 2 min. 30 sec. and to three more beginning at 3 min. 10 sec. In some other cases responses occurred to successive stimuli in this way, but in general such results are exceptional, as will be seen from statistics to be presented later.

The size of the responses to single stimuli deserves some comment. That the size of the individual contractions is variable is perfectly clear from the records, and this has been the experience of the unrecorded tests as well. No maximal contractions have been recorded. In the unrecorded tests a preponderance of powerful over small contractions has been indicated, but, of course, in the absence of records it is not possible to be certain of this. What is clear is that the upper limit in the size of the responses to single stimuli seems to lie within the range given by pairs of stimuli within the facilitation period. The implications of this are important and will be considered later on.

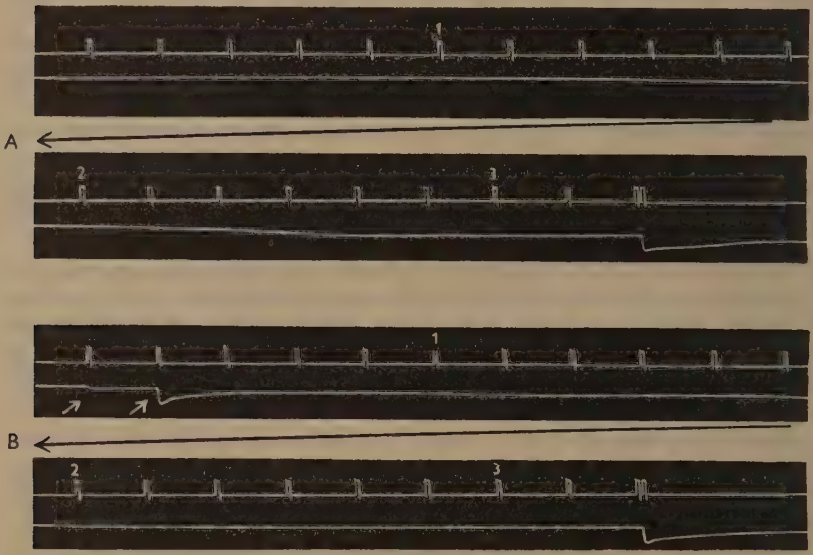

Fig. 1. A (control). *Metridium* stimulated every 10 sec. for 3 min. 10 sec. with quick response to a pair of stimuli 1 sec. apart at 3 min. 20 sec. B (treatment). The same animal stimulated as above after introducing *Metridium* extract into the sea water (dose: 0·002 M units per 100 c.c.). Arrows indicate quick responses to single stimuli; signals represent stimuli and times from beginning of control and treatment. (See p. 243 for definition of M unit).

These records illustrate one noteworthy feature of the results obtained from both recorded and unrecorded tests. This is the comparative infrequency of quick responses elicited directly by the extract. Such direct effects would take the form of quick closing movements when the extract was introduced or between the stimuli in the series. Figures to be presented later will show that such spontaneous movements are absent in the vast majority of tests, a fact of some significance in determining the origin and significance of the effects.

There are other direct effects that occur more frequently during the treatments, but which do not show up on the smoked records. Thus some twitching and withdrawal of the tentacles usually occurs as soon as the extract is introduced. With weak doses this ceases between 30 sec. and 1 min. With stronger doses the tentacles may remain withdrawn and a slow retraction of the whole animal may occur producing a state of partial closure. As a rule this condition is not maintained longer than 2 or 3 min., and unless closure occurs in response to the electrical stimuli, the anemone is found in the expanded condition at the end of the treatment. The records show also that at the end of the treatment the response to a pair of stimuli 1 sec. apart is virtually unchanged in size and form, and it can be established that there is no effect on the duration of the facilitated state.

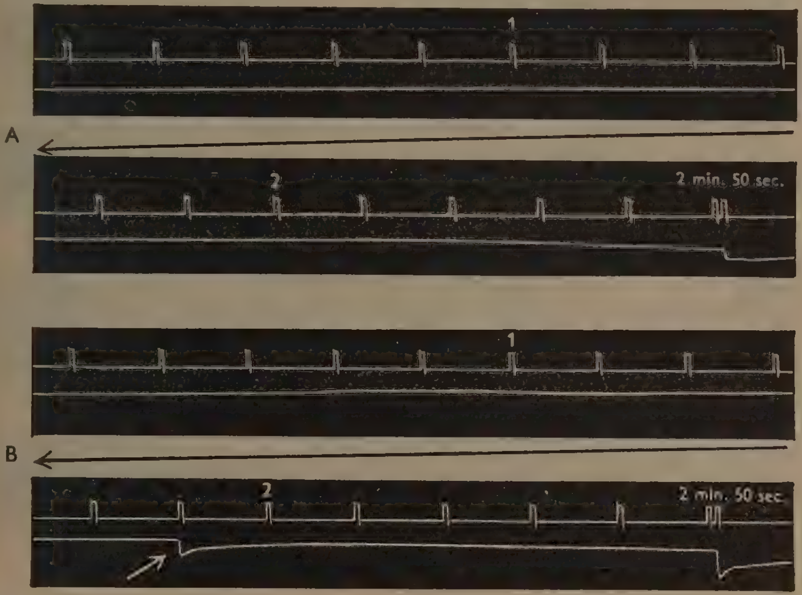

Fig. 2. A (control). *Metridium* stimulated every 10 sec. for 2 min. 40 sec. with quick response to a pair of stimuli 1 sec. apart at 2 min. 50 sec. B (treatment). The same animal stimulated as above, after introducing of *Metridium* extract into the sea water (dose: 0·018 M units per 100 c.c.). Arrow indicates quick response to single stimulus; signals represent stimuli and times from beginning of control and treatment.

Frequency of responses to single stimuli in untreated animals

For most practical purposes it can be assumed that at ordinary temperatures *Metridium* and *Calliactis* never give quick responses to single stimuli or to individual stimuli in a series more than about 3 sec. apart. This is not strictly true. Untreated animals occasionally do respond to single stimuli or to occasional stimuli in a low-frequency series of the kind employed in these tests. A simple explanation for such responses is that the effective single stimulus follows closely on or precedes an impulse set up in the nerve net by ordinary sensory stimulation. Because the effect of the extract usually takes the form of occasional quick responses in such a series, it is desirable to establish exactly how often quick contractions occur in untreated animals and to compare this with the frequency of such responses in animals treated with extracts.

Data on the frequency of quick responses in untreated animals can be obtained from the controls carried out in the tests. Early in the work a series of ninety-six tests were carried out in which five *Metridium* extracts were tested on *Metridium* over a moderate dosage range, and from this series many of the quantitative data about the responses to single stimuli have been derived. In the controls of these ninety-six tests a total of eight quick contractions occurred to 1400 stimuli, i.e. a frequency of 1 in 175. A further 179 controls were carried out in connexion with other work, and in these eleven quick contractions occurred to 3136 stimuli, i.e. a frequency of 1 in 285.

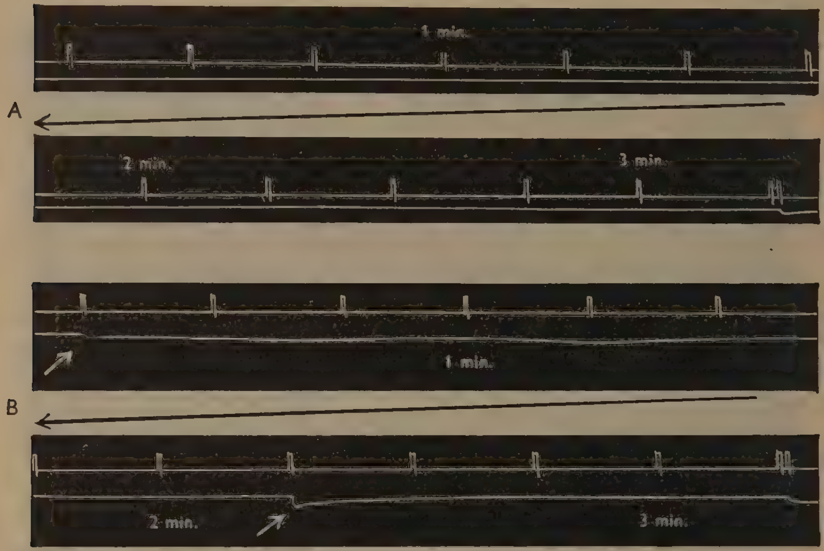

Fig. 3. A (control). *Metridium* stimulated every 15 sec. for 3 min. with quick response to a pair of stimuli 1 sec. apart at 3 min. 15 sec. B (treatment). The same animal stimulated as above after introduction of *Metridium* extract into the sea water (dose: 0·018 M units per 100 c.c.). Arrows indicate responses to single stimuli; signals represent stimuli and times from beginning of control and treatment.

Information on this point comes also from experiments in which *Metridium* was stimulated for long periods at frequencies of 5, 10 and 30 sec. A typical record of such an experiment is shown in Fig. 5, where a stimulus was given every 10 sec. for 24 hr. The quick responses can be recognized as sudden downward movements of the lever, all the smaller movements being slow and almost imperceptible movements of the animal. Not more than thirteen of the movements on this record can be typical quick contractions. Assuming that all these are in response to thirteen out of the 8640 electrical stimuli delivered in 24 hr., this experiment shows a frequency of one quick response for 665 stimuli in an untreated animal. Data from this and other experiments of the same type are summarized in Table 1. Although there is considerable variation in the frequency of such responses in the different experiments, it never rises above 1 per 100 stimuli and the average figure is 1 in 400.

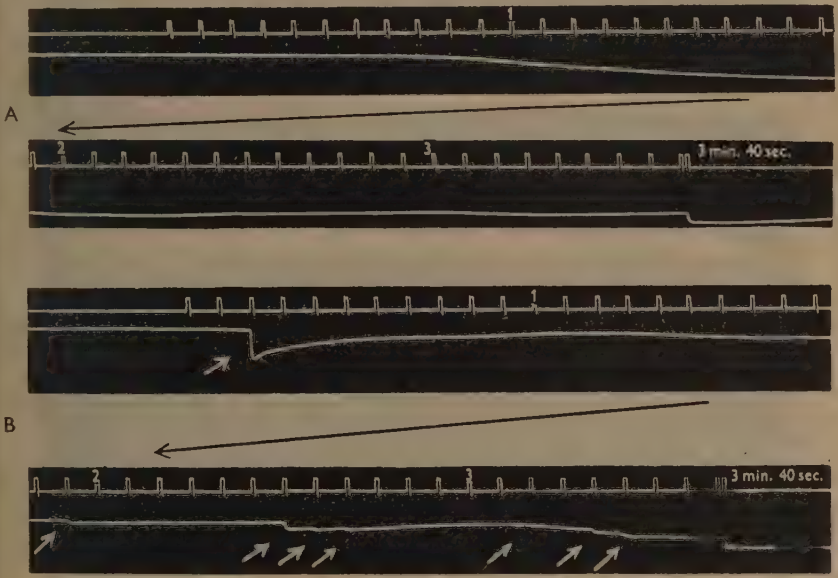

Fig. 4. A (control). *Metridium* stimulated every 5 sec. for 3 min. 30 sec. with quick response to a pair of stimuli 1 sec. apart at 3 min. 40 sec. B (treatment). The same animal stimulated as above after introduction of *Metridium* extract into the sea water (dose: 0·018 M units per 100 c.c.). Arrows indicate responses to single stimuli; signals represent stimuli and times from beginning of control and treatment.

There is a fair measure of agreement between both these sets of results, from the controls of the tests and from long-continued stimulation. Considering all these data together one can conclude that a normal untreated *Metridium* requires of the order of hundreds of single or low-frequency stimuli for each quick response that occurs.

Frequency of quick responses following treatment with extracts

The same ninety-six tests whose controls were referred to in the preceding section provide the essential data for estimating the frequency of quick responses following the treatment. These tests were all carried out with moderate doses, and the extracts used were all ordinary crude extracts tested in a routine manner. To the 1400 stimuli given to animals in the course of these ninety-six tests, 155 quick responses were obtained. This is a frequency of 1 in 9 compared with 1 in 175 in the controls of the same tests. Thus the effect of the extract may be expressed statistically as a 20-fold increase in the probability of a quick contraction in the course of low-frequency stimulation lasting 3 min. It is useful also to consider the data from these ninety-six tests from the standpoint of the number of quick responses observed per control and per treatment. Table 2 sets out the results in this way, and shows that whereas quick responses occurred in only one-thirteenth of the controls, they occurred in five out of every six treatments. Exactly two-thirds of the treatments gave only one or two responses and only the remaining one-sixth (16) gave three or more. The highest number of responses observed in the course of a single treatment was ten.

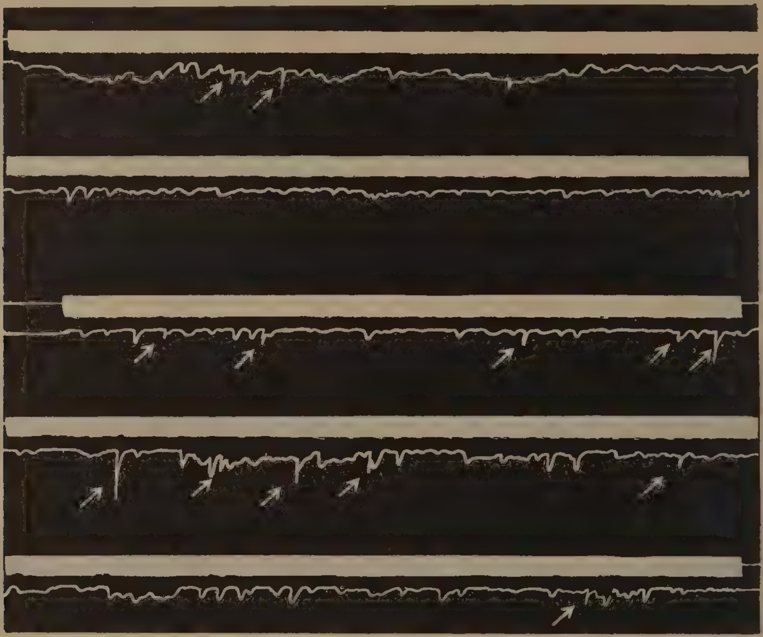

Fig. 5. *Metridium* stimulated every 10 sec. for 24 hr. Arrows indicate the thirteen quick responses recorded to the 8640 stimuli given. All other movements of the lever are slow responses.

Table 1. *Frequency of quick responses in untreated animals*

Metridium stimulated every 5, 10 or 30 sec. for periods up to 24 hr.

Experiment ...	I	II	III	IV	V
Duration	3 hr.	3 hr.	6 hr.	24 hr.	24 hr.
Frequency of stimulation	5 sec.	5 sec.	10 sec.	10 sec.	30 sec.
Total stimuli	2160	2160	2160	8640	2880
Quick responses	4	20	2	13	6
Stimuli per response	540	108	1080	665	480

Table 2. *Comparison of controls and treatments according to number of responses in tests lasting 3 min.*

Responses during 3 min. stimulation	No. of controls and treatments with 0–10 quick responses in ninety-six tests with *Metridium* extracts. (Each test, stimulated every 10 sec. for 3 min.)	
	Controls	treatments
0	89	16
1	6	38
2	1	26
3	0	11
4	0	1
5	0	1
6	0	1
7	0	1
8	0	0
9	0	0
10	0	1
Total responses	8	155

The potency of the extracts

The extracts were prepared from five or ten moderately large *Metridium* or *Calliactis*. Volumetric samples introduced in the tests therefore represent fractions of the material extracted from these animals. For want of a more precise quantity, an arbitrary unit representing the material extracted from a single anemone will be used in comparing the effects of different doses and these will be termed M (*Metridium*) and C (*Calliactis*) units. These tests were usually done in 50 or 100 c.c. containers using small *Metridium* as the test animals, a measured sample of concentrate being run in rapidly to ensure thorough mixing with the sea water bathing the animal. It is convenient to express doses as M or C units per 100 c.c.

Preliminary work confirmed the rough observation reported in the earlier work (Ross, 1945*b*) that the introduction of samples containing less than one-tenth of the material extracted from a single anemone was followed by the occurrence of responses to single stimuli on occasion. It was important in comparing the potency of different extracts to find out what is the effect of varying the dose and especially to determine the minimum effective dose as accurately as possible. The number of responses occurring to stimuli at a frequency of one in 10 sec. in ten tests lasting 3 min. was taken as a measure of the effectiveness or potency of a given treatment.

Fig. 6 summarizes in graphical form the number of responses occurring in ten such tests with three different extracts of *Metridium* over a dosage range of 0·1–0·0045 M units in 100 c.c. of sea water. Thus each point on the graph represents the number of responses obtained when 180 stimuli are given. There is clearly a close relationship between the quantity of extract introduced and the number of responses obtained and a fair measure of agreement in the dose-potency curves given by the three different extracts. Thus all three extracts agree in showing over twenty quick responses per ten treatments with 0·1 M units and less than five responses below 0·005 M units. This lower figure approaches the frequency of responses in untreated animals (1 in 175 in the controls of the ninety-six tests) and must therefore be considered as close to the minimum effective dose.

A noteworthy feature of the potency-dose curve is represented in Fig. 6 by the tests with extract 4 in which doses were reduced by small amounts over the range 0·0075–0·004 M units. Here there is evidence of a sharp falling off in the frequency of quick responses, a result that is highly suggestive of the approach to a threshold dose.

The absolute quantities represented by the doses of extract used in these tests are small. The dry weight of the solid matter contained in samples of extract 4 was determined and has given a figure of 680 mg. as the weight of an M unit in this case.

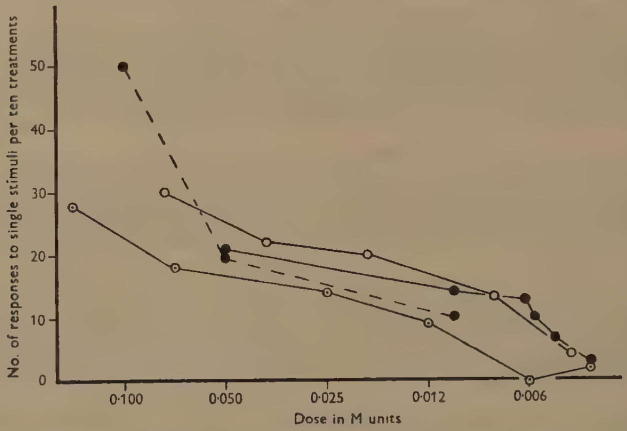

Fig. 6. Potency-dose graphs for three *Metridium* extracts. ⊙, *Metridium* extract 1; ○, *Metridium* extract 2; ●, *Metridium* extract 4. (Broken and complete lines represent separate series of tests.)

Thus the quantities of extracted substances contained in doses from 0·1 down to 0·004 M units range from 68 to 2·7 mg. per 100 c.c. These are equivalent to dilutions of solid substance in sea water of 1 : 1500 to 1 : 37,000. If as seems likely, the substance (or substances) responsible for inducing these responses to single stimuli forms only a small proportion of the extracted matter, the active principle in the extract is probably exerting its effects over a dilution range of hundreds of thousands to millions of parts in sea water. Clearly it is a sensitive reaction, though less so than the direct response of certain isolated effectors in other animals to substances of great activity like acetylocholine.

The extracts whose potency we have just considered were obtained from animals stimulated intensely by the handling and cutting involved. Attempts were made to prepare extracts from unstimulated animals for comparison with the ordinary extracts. One method used was to chill the animals to −2° C. and anaesthetize with $MgCl_2$ (0·4 M), treatments which enable animals to be handled and cut up with little or no muscular response. Another method was to 'quick-freeze' the anemones with liquid air, a treatment which fixes them in the expanded condition and must kill them almost instantaneously.

Fig. 7 sets out the results of tests of such extracts as potency-dose graphs, with one ordinary extract from Fig. 6 for comparison. These results show no significant difference between the activity of extracts from 'stimulated' and 'unstimulated' animals; the number of responses per ten treatments is of the same order over the range of doses examined. The results of tests on 'quick-frozen' animals are less conclusive, but at the near-threshold doses, to which perhaps more importance should be attached, such extracts appear to display the normal degree of activity.

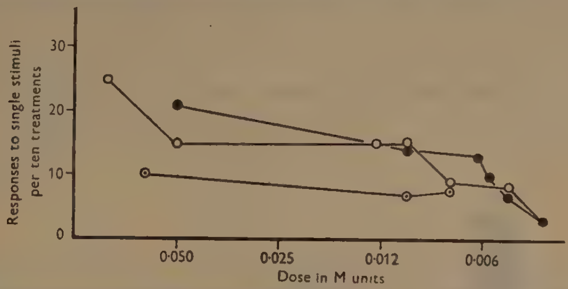

Fig. 7. Potency-dose graphs for extracts from 'stimulated' and 'unstimulated' animals. ⊙, extract from quick-frozen animals; ○, extract from anaesthetized animals; ●, ordinary extract 4 for comparison.

Table 3. *Activity of extracts from the sphincter-disk region compared with extracts from below the sphincter.*

Dose (C or M units per 100 c.c.)	Responses per 10 treatments (stimuli at 1 per 10 sec. for 3 min.)			
	Metridium		*Calliactis*	
	'Sphincter disk'	'Sub-sphincter'	'Sphincter disk'	'Sub-sphincter'
0·05	18	7	14	3
0·01	17	7	—	—
0·0075	15	8	—	—
0·005	13	5	11	6
0·003	13	0	—	—
0·001	4	2	—	—

Some tests on extracts of *Calliactis* were also carried out. It was confirmed that these caused responses to single stimuli exactly like the *Metridium* extracts. The potency-dose curves were similar in form, but the minimum effective dose is slightly lower, between 0·003 and 0·001 C units.

In both *Metridium* and *Calliactis* separate extracts were prepared from animals divided into 'sphincter-disk' (includes tentacles, disk and marginal sphincter) and subsphincter' fractions, and the activity of the two fractions compared. Table 3 presents the results and shows that the extracts from the sphincter-disk fraction are consistently more effective in producing responses to single stimuli. The activity of the sphincter-disk extracts is relatively even greater than these figures indicate because this fraction contains only about one-quarter of the total solid in *Metridium* and one-third in *Calliactis* (estimates based on wet weights of residues after filtering the crude extract). It is clear that whatever the nature of the presensitizing substance and its role in the living anemone, it is present in much larger quantities in the disk, tentacles and sphincter than in the rest of the anemone. Perhaps it is worth noting that this division would probably correspond with a division based on nematocyst distribution.

Temporal aspects of the responses to single stimuli

The distribution of the quick responses over the 3 min. period following the introduction of the extract in the ninety-six standard tests is shown in Fig. 8. There is a pronounced tendency for the responses to occur early in the treatment, almost one-half of the responses occurring in the first minute and less than one-fifth in the third minute of the treatment. Further breakdown of these figures has shown that a large proportion (30 out of 155) occurred in the quarter-minute between 15 and 30 sec. after the extract sample was introduced. This underlines the rapidity with which the effect makes its appearance, a feature which was not so evident in the earlier results and a point of some importance in considering the nature of the effects.

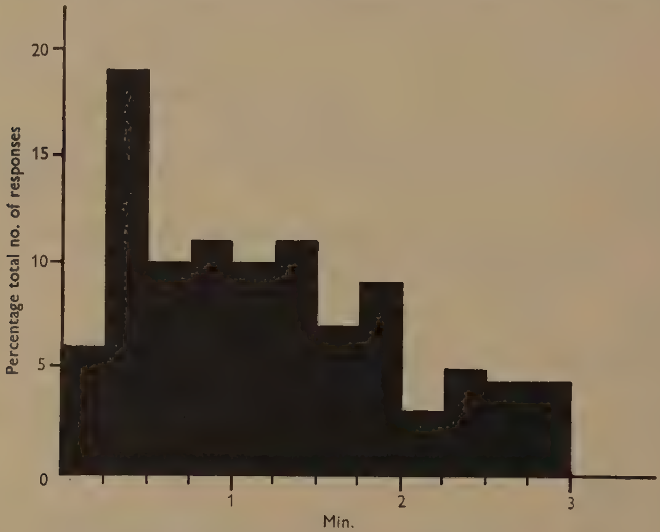

Fig. 8. Time distribution of responses to single stimuli in 3 min. tests.

Further work has shown that there is some relation between the time of appearance of the quick responses and the amount of extract introduced. Table 4 brings out this fact, showing that as one passes down the dosage range from 0·5 to 0·005 M units, the proportion of the responses occurring early in the 3 min. tests falls off, while the proportion in the later stages of the treatment rises sharply. This is perhaps what one would expect on *a priori* grounds assuming that the effect reflects the rise of a substance in solution to a threshold concentration, the threshold being reached earlier the higher the dose introduced.

Perhaps the most characteristic temporal feature of the effect is its sporadic and discontinuous character. Thus it is unusual for responses to occur to successive stimuli in a series. If we examine the data from the ninety-six standard tests, we find that out of the 155 quick responses obtained in these tests, 129 occurred to isolated stimuli. Only 7 pairs of responses to two successive stimuli and only four groups of three responses to three successive stimuli were observed. Clearly the condition set up by the extract, which enables a single stimulus to cause a response, is generally of short duration, in most cases not lasting longer than 10 sec. if one takes the data from these tests at their face value. Four of the other tests with extracts, not included in the ninety-six standard series, have produced responses to four successive stimuli; on two other occasions responses to five successive stimuli were observed. Yet the overall picture is the same, suggesting a condition that comes and goes during the treatment, and perhaps does not outlast the ordinary decay period of facilitation. This aspect of the results must assume considerable importance in interpreting the effects.

Table 4. *Proportion responses early and late in treatments with different doses of* Metridium *extract*

Dose (M units/100 c.c.)	Percentage responses at different stages of 3 min. treatments		
	0–1 min.	1–2 min.	2–3 min.
0·05	91	9	0
0·01	89	0	11
0·006	60	40	0
0·005	29	23	48

RESPONSES TO SINGLE STIMULI WITH OTHER TREATMENTS

The observation that extracts of *Metridium* and *Calliactis* enable responses to occur to occasional shocks in a low-frequency series so soon after being introduced, led to the re-examination of the effects of other treatments studied previously over a longer time scale. This matter has not been exhaustively investigated, but enough has been done to establish the fact that a variety of treatments can cause responses which are apparently similar to the responses to single stimuli which follow the extract treatments.

Fig. 9 illustrates the effect on *Metridium* of suddenly increasing the acidity of the sea water to pH 3·0 and the occurrence of a response at 20 sec. Fig. 10 shows records taken before and after treatment with sodium taurocholate (1 : 200) in which several responses to single stimuli may be seen. Table 5 summarizes the results of these and other treatments according to the number of responses obtained per ten tests. It seems that most treatments have some effects in this respect, but usually only at high concentrations. In certain cases the effect is very

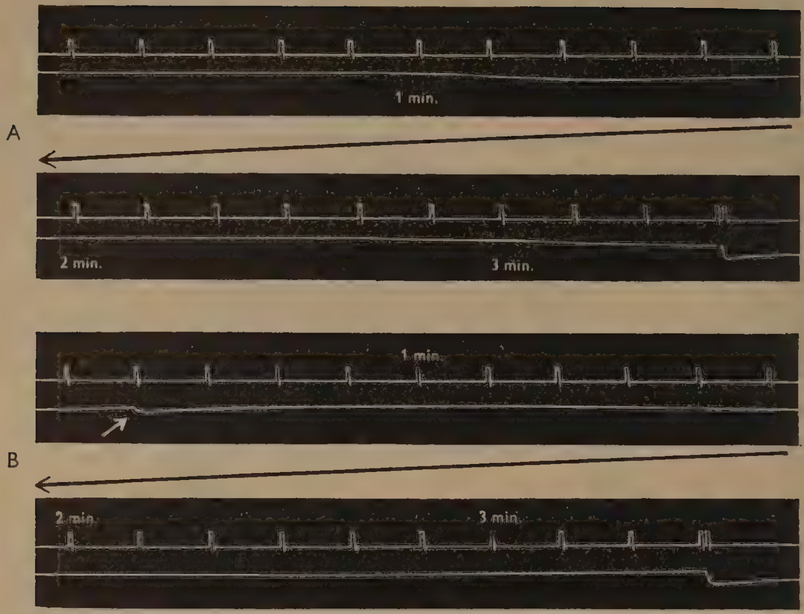

Fig. 9. A (control). *Metridium* stimulated every 10 sec. for 3 min. 20 sec. with quick response to a pair of stimuli 1 sec. apart at 3 min. 30 sec. B (treatment). The same animal stimulated as above after the addition of H_2SO_4 bringing the sea water to pH 3·0. Arrows indicate responses to single stimuli; signals represent stimuli and times from beginning of control and treatment.

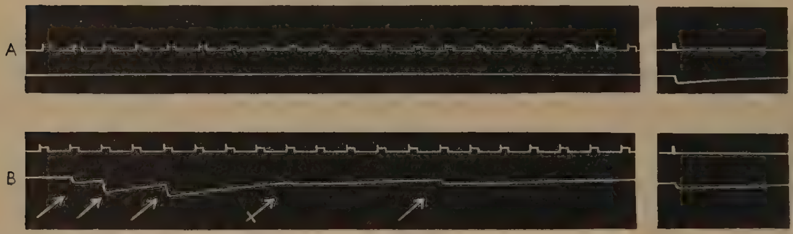

Fig. 10. A (control). *Metridium* stimulated every 9 sec. for 3 min. with quick response to a pair of stimuli (0·4 sec. apart) 1 min. later. B (treatment). The same animal stimulated as above after the addition of Na taurocholate (1 in 200 parts sea water) with quick response to a pair of stimuli (0·4 sec. apart) 1 min. later. Ordinary arrows indicate 4 responses to single stimuli; crossed arrow a delayed response; signals represent stimuli and times from beginning of control and treatment.

slight. This is particularly so with some of the drugs which have the most pronounced neuro-muscular effects elsewhere, acetylcholine, histamine, etc. No substances of this type approach the activity of the extracts which gave upwards of twenty responses per ten tests with higher doses. The ineffectiveness of tyramine and the relatively slight action of 933 F are noteworthy here, in view of the effects of these substances in increasing the size of the quick responses to pairs of stimuli and causing responses to single stimuli in *Calliactis* after 1–3 hr. treatment (Ross, 1945 *a*).

Table 5. *Responses to single stimuli in standard tests with various reagents, drugs and other treatments*

Treatment	Dose	Presensitized responses per ten tests	
		Controls	Treatments
Acid: H_2SO_4	pH 3·0	0	13
H_2SO_4	pH 6·4	1	1
HCl	pH 2·4	0	12
Alkali: –NaOH	pH > 10	1	9
–NaOH	pH 9·2	0	1
NH_4OH	pH > 10	0	6
Salts: KCl (0·6 M)	1 : 9 sea water	1	21
KCl (0·6 M)	1 : 19 sea water	0	3
$CaCl_2$ (0·4 M)	1 : 9 sea water	2	4
NH_4Cl	1 : 340 sea water	0	10
NH_4Cl	1 : 1700 sea water	0	0
Drugs, etc.:			
Acetylcholine	1 : 100	0	3
Adrenaline	1 : 10000	0	7
	1 : 100000	0	0
Tyramine	1 : 1000	0	0
	1 : 10000	1	0
Histamine	1 : 1000	0	6
	1 : 2500	0	2
	1 : 10000	0	1
Cocaine	1 : 1000	0	0
933 F	1 : 10000	0	4
Ethylamine	1 : 1000	1	1
Triethylamine	1 : 1000	0	1
$(CH_3)_4NOH$	1 : 100	0	8
	1 : 200	1	6
	1 : 1000	0	1
$(CH_3)_4NI$	1 : 1000	0	5
Surface-active substances:			
Sodium taurocholate	1 : 200	1	32
	1 : 1000	0	4
Saponin	1 : 500	0	36
*Animal juices:			
Squid	1 : 100	0	2
	1 : 1000	0	0
Mussel	1 : 100	0	1
	1 : 1000	1	1

* Concentrated extracts from *Loligo* skin scrapings and minced *Mytilus* muscle respectively.

The reagents which have the most pronounced effects here are strong acid and alkali, potassium chloride ($K^+ \times 8$) and especially bile salt and saponin. These are drastic treatments which might be expected to have widespread non-specific effects on a whole animal. Yet if one compares this list with Pantin's (1942) list of substances causing the nematocysts in the tentacle of *Anemonia* to discharge, it becomes apparent that treatments of the same character are involved in both cases. In this respect the special activity of bile salt must be regarded as highly suggestive.

The effects of these treatments do not differ in any obvious way from the effects observed with extracts. The responses tend to occur more frequently in the first minute of the treatment. They generally occur to isolated rather than successive stimuli; in other words, the effect is usually sporadic and of short duration. Nevertheless, a number of remarkable results were observed in some of the tests with the more effective treatments, especially bile salts, saponin and KCl. These results showed that it is possible with these reagents, as with extracts on occasion, to obtain quick responses from as many as four successive stimuli 10 sec. apart, so the effect is not invariably short-lived.

The effects of these treatments are not accompanied by an appreciable number of spontaneous contractions, even at the relatively high doses here employed. If much higher doses than these are used, the introduction of the reagent often causes an immediate and complete closure of the animal and re-opening may not occur. It is noteworthy that, as with extracts, there seems to be no transitional phase in which a high proportion of responses to single stimuli grades into increasing degrees of spontaneous activity. The latter has a distinct all-or-nothing character which is very striking indeed.

With the exception of $K^+ \times 8$, bile salt and saponin, the activity of these reagents is generally far below that of the extract treatments. There is certainly no possibility that the extract effects can be attributed to any of the substances here tested. Tetramethylammonium hydroxide, identified in extracts of *Actinia equina* by Ackermann, Holtz & Reinwein (1923) and suggested by them as the poison of the nematocysts, is only active at concentrations far above any it could possibly have in the extract treatments. Moreover, the doses employed to induce the effects are too strong for it to be suggested that any of these substances could be involved in the physiological processes of the quick response in the normal animal. There is a contrast here with the extract effects, for we have seen already that any active principle in the extract is probably diluted hundreds of thousands of times in the treatments over the moderate dosage range.

DISCUSSION

It seemed from the earlier work with extracts (Ross, 1945*b*) that the presence of a substance in extracts of sea anemones which enabled single stimuli to cause responses lent considerable support to the notion that facilitation is a chemical process and the active substance in the extract a 'facilitator' in the anemone itself. This view may have reflected an unduly strong influence of the concept of chemical transmission on the interpretation of those results. At any rate, for a number of reasons, the view that the effects of the extracts can be attributed to a 'facilitator' now seems unaceptable.

The demonstration that such effects can occur with other treatments besides the extracts would not by itself be inconsistent with a chemical interpretation of facilitation. Indeed, if facilitation were a chemical process, it would be surprising if certain substances with widespread effects on living tissues did not have a facilitating action, especially when employed in powerful doses. Nevertheless, the fact that similar effects can be produced by so many different treatments makes it necessary to be cautious before regarding the extract effect as anything more than a general response to substances not normally present in the sea water.

The results of the potency tests on extracts from 'unstimulated' animals are relevant in this connexion. If the responses to single stimuli were due to a 'facilitator' it would seem reasonable to expect that the activity of such extracts, even if not completely abolished, would at least be substantially depressed. The results, however, provided no evidence that the extracts from anaesthetized or quick-frozen animals were less potent than extracts prepared in the ordinary way. It therefore seems unlikely that there is any connexion between the active substance (or substances) in the extracts and the processes of stimulation and response in the normal animal.

Finally, the consistently early appearance of the effects, as early as 10 sec. after the introduction of the extract, has some bearing on this matter. It is hardly credible that in such a short space of time the active substance could enter the tissues of the animal and reach the junctions where the facilitation process is apparently carried out. This feature of the effect is much more in keeping with some superficial action by which the extract sets up facilitation indirectly and not by itself acting as a 'facilitator'.

If the responses to single stimuli are not due to a direct facilitating action, then we must look for their origin in excitation conducted to the muscles through the nerve net. There is overwhelming evidence in Pantin's papers (1935 *a–d*) that general excitation in the nerve net takes the form of discrete impulses in the through-conduction tracts, impulses whose frequency determines which muscles will be brought into operation. We can apply this fact to interpret the effects described in the foregoing account.

It will be obvious that a response will occur whenever a single electrical stimulus happens to be preceded or followed by an adventitious impulse in the through-conduction tracts. Of course such a response would not be a response to a single impulse at all but a response to the second of a pair of impulses. This is how one tends to explain the few quick responses which occur in untreated animals (p. 239). Is it possible that the effects of the treatments simply reflect the passage of occasional impulses in the nerve net? Is it possible in fact that in these results we see the effect of increasing the frequency of an event which is going on all the time in the normal animal at a very low frequency indeed?

The evidence does not yet allow an unqualified answer to be given to either of these questions, but there are certain important features of the results that are consistent with affirmative answers to both. Thus one cannot fail to be impressed with the sporadicity of the responses to single stimuli observed in these tests. Figures cited above have shown that the majority of these responses occurred in response to isolated and not to successive stimuli. Although up to five responses to successive stimuli have been observed, it remains true that in any given test the responses seem to occur in a random and discontinuous fashion. Both these features of the effects are in keeping with the conception that the treatments are setting up occasional impulses which now and then are close enough to some of the electrical stimuli for apparent responses to occur to the latter.

Again one must note the variability in the size of the responses to single electrical stimuli that have been observed in the tests. We have seen that the size of the individual responses generally varies between the range given by pairs of stimuli in the normal animal. This, too, is consistent with the view that the response to a single electrical stimulus occurs when it is accompanied by an adventitious impulse within the normal facilitation period.

There are several other features of the observations that are, however, less consistent with this view, at any rate in this simple form. If the effect of the treatments is to set up impulses in the general nerve net similar to those set up by electrical or mechanical stimuli it would seem reasonable to expect a good deal of spontaneous activity. After all, whenever two or more impulses are close enough together (2 sec. or less), spontaneous quick responses should occur in the absence of electrical stimuli altogether. However, such effects are rare. In the ninety-six tests which yielded 155 responses to single electrical stimuli only ten spontaneous contractions were observed, most of them (seven) when the extract was being run in, or immediately after, when any response might equally well be due to mechanical stimulation as to direct chemical effects. Thus if the responses to single stimuli are to be ascribed to adventitious impulses set up by the treatments, it must be assumed that these impulses are generally too far apart to cause quick contractions by themselves.

There is indeed some evidence that treatments of this kind do set up low frequency excitation waves in the nerve net. Pantin (1935*b*, 1950) has shown that certain of the slow reflex movements, which can be elicited by food substances and other chemicals, can be obtained by electrical stimuli at frequencies outside the range which brings in the quick response. Yet if low-frequency impulses of this kind are responsible for the effects observed in these experiments, it is puzzling that food stimulants like molluscan juices, which presumably set up low-frequency excitation, do not cause responses to single stimuli (Table 5). Evidently one must bear in mind that there are qualitative differences in the excitation set up by different kinds of stimuli as well as differences in the frequency of impulses set up in the general nerve net.

The results show a second curious feature. If the responses to single stimuli are due to adventitious impulses which happen to occur close to some of the electrical stimuli, one would expect such adventitious impulses to follow the electrical stimuli just as often as they precede them. Thus, in a large number of experiments, one should get about equal numbers of responses 'on the stimulus' and responses which follow the stimulus only after a delay of anything up to 2–3 sec. This expectation is not fulfilled. Delayed responses do occur from time to time (cf. Fig. 10 for an example), but they are remarkably infrequent. In the ninety-six standard tests 155 responses were obtained, all of them on or very close to the stimuli. In the same tests only five delayed responses were observed. In the other tests the number of visibly delayed responses was equally insignificant. If adventitious or low-frequency impulses are indeed responsible for the responses to single stimuli, it is surprising that they should so often precede, and so seldom follow, the electrical stimuli. The matter can be explained, of course, by ascribing exceptional properties of refractoriness to those parts of the nerve net in which the adventitious impulses arise, or by attributing inhibitory effects to the electrical stimuli. Yet to suggest that is to imply that the responses to single stimuli are associated with a special part or activity of the nerve net, a part whose properties differ from the through-conducting system. In that case one cannot assume that the excitation set up by such an activity is identical in scope or character with the general excitation of the through-conduction tracts.

To conclude. It can be stated that the general character of the responses to single stimuli is in keeping with the insertion of extra impulses into the series set up by the electrical stimuli employed in the tests. At the same time, the capacity of these impulses to set up facilitation contrasts sharply with their incapacity to call forth the quick response, judging from the infrequency of spontaneous and delayed contractions. This might be due to the low frequency of such impulses and to special refractory or inhibitory properties in the sensory or nervous elements involved. Alternatively, it might reflect a genuine incapacity to initiate the contractile phase of the response on the part of impulses set up by the treatments. That would imply separate processes or channels of excitation, responsible separately for the facilitating and excitant phases of the response. It is worth recalling in this connexion that earlier work, especially on ions and drugs, emphasized that the two processes involved in the quick response—facilitation and excitation—are essentially independent (Pantin, 1937; Ross & Pantin, 1940; Ross, 1945*a*). Perhaps the possibility of distinct facilitating and exciting modes of local excitation in the neuro-muscular units taking part in this response is one that should be borne in mind in future work.

SUMMARY

1. The previously reported effect of anemone extracts, the occurrence of quick closing responses to single electrical stimuli in *Metridium*, has been re-investigated. In standardized tests it was found that whereas hundreds of stimuli are required for each response to a single stimulus in untreated animals, after anemone extract the incidence of such responses is one per nine stimuli.

2. The incidence of these responses falls off with decreasing doses of extract and the effect disappears when less than $\frac{1}{500}$th of the material from a single large *Metridium* is administered. There is no evidence that extracts from 'stimulated' and 'unstimulated' (i.e. anaesthetized or quick-frozen) anemones differ in potency. Extracts from divided animals show greater activity in the 'sphincter-disk' fraction.

3. The incidence of the responses also falls off in time and is highest from 15 to 30 sec. after beginning the treatment. The effect is sporadic and short-lived and responses to two or more successive stimuli are exceptional.

4. A number of treatments, such as drastic changes in pH, KCl($K^+ \times 8$), tetramethylammonium hydroxide (1 : 100), NH_4Cl (1 : 340) and especially bile salt

and saponin, have similar effects. Drugs with neuro-muscular effects elsewhere (acetylcholine, adrenaline, tyramine, histamine, etc.) were generally ineffective except at very high doses. Food stimulants too were ineffective.

5. From the time relations and other aspects of the responses to single stimuli it is concluded that the effect should not be attributed to a substance with the function of a 'facilitator' in the living animal.

6. While the effects are consistent with the passage of occasional adventitious impulses in the nerve net, there is a singular absence of spontaneous or post-stimulus contractions. Certain implications of this feature of the results are discussed.

It is a pleasure to acknowledge a special obligation to Mr Graham Hoyle who, during the final year of an Honours Course, assisted me by carrying out some hundreds of tests on anemone extracts and other substances. Data from these tests provided a major part of the information on the potency of extracts from stimulated, unstimulated and divided animals, and the discussions we had together were of real value to me. I am also much obliged to the Director and Staff of the Plymouth Laboratory for facilities enjoyed in August and September 1948, to Mr G. P. Wells, Dr Bernhard Katz and Mr D. R. Newth, who read the paper and offered many valuable criticisms and suggestions, and to Dr C. F. A. Pantin, F.R.S., for the privilege of consulting him from time to time.

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OBSERVATIONS ON THE MECHANISM OF FEEDING IN THE SUCTORIAN *PODOPHRYA*

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(*Received* 16 *July* 1951)

(With Plates 10 and 11 and Four Text-figures)

INTRODUCTION

Most Suctoria catch their prey by means of tentacles, to the tips of which the prey adheres. The contents of the prey then pass up the tentacles into the body of the captor. It is the purpose of this paper to present additional data bearing on this process of feeding.

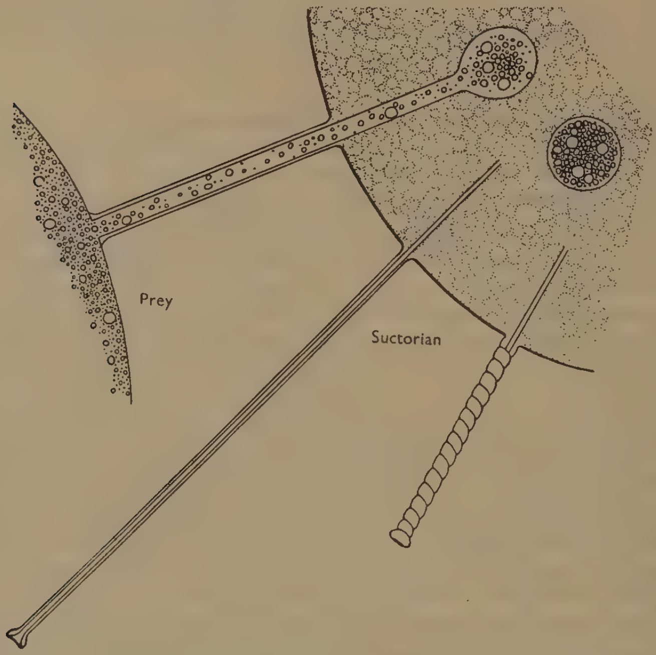
The tentacles of a suctorian are described as consisting of two concentric tubes (Collin, 1912), the walls of which unite at the distal end. The wall of the outer tube appears to originate in and to be continuous with some component of the body surface of the suctorian (Noble, 1932). The inner tube projects inwards from the tentacle for a short distance into the cytoplasm, and ends abruptly. During feeding the contents of the prey pass through the inner tube. These relations are illustrated in Text-fig. 1, reproduced from Noble's paper (Noble, 1932). Possibly the space between the two tubes, which is continuous with the ectoplasm of the suctorian, is occupied by gelated protoplasm having considerable structural rigidity. The elongated shape could not be maintained without some rigidity, and the disintegration of suctorian tentacles under the influence of high hydrostatic pressure (Kitching & Pease, 1939) suggests the collapse of a gel structure. According to Kormos (1938) the tentacles emerge through holes in the cuticle of the suctorian.

The tentacles terminate, at their distal ends, either in a knob or in an open funnel. Capture of prey is attributed by some authors to the development of a sudden suction. However, according to Kormos (1938) tentacles with open funnels are incapable of capturing live prey; he attributes capture to a stickiness of the knob.

There is evidence to suggest that the body of the prey may be subject to some digestive influence while the suctorian is feeding upon it. Iziumov (1947) coloured the ciliate *Glaucoma* with congo red and then offered it to the suctorian *Tokophrya infusionum*. A change in colour, indicating increased acidity, was observed in the prey in the region where the tentacles of the suctorian were attached. He also observed the dissolution of grains of paramylum in a captured *Chilomonas*.

The mechanism of the suction of Suctoria has provoked much speculation. Early work is summarized in Collin's fine monograph (Collin, 1912), which contains full references. Collin rejected the idea that material flows along the tentacles of the suctorian because of the hydrostatic pressure within the body of the prey;

but this explanation has more recently been reaffirmed by Pestel (1931). Collin (1912) considered that the pressure required would be too great, and believed that the liquid contents of the prey were driven along by peristalsis of the inner tube of the tentacle. Other authors refer to suction but do not explain it. Kahl (1931) rejected suction on the grounds that it would involve an expansion of the surface, which he was unable to believe in.



Text-fig. 1. Parts of a suctorian and its prey, showing the structure of the tentacles. (Reproduced from Noble, 1932, fig. 4.)

MATERIAL AND METHODS

Podophrya sp. was used in this work. I have not seen any cysts, but this species resembles *P. collini* Root (1915) in appearance and type of prey captured. It was originally found in a pond in the University grounds, and was maintained in culture. *Podophrya* has a pear-shaped or spheroidal body, which is attached to the substrate by a stalk. The tentacles are born on all parts of the body surface, and are swollen at the tips (see Pl. 10). The body surface is normally smooth in outline and free from dents or wrinkles. However, some specimens cultured in 10 % sea water were wrinkled.

Podophrya sp. was cultured in closed glass dishes and grown on silk threads as already described (Kitching, 1951). The culture medium was Bristol tap water, or for certain experiments 5 or 10 % sea water, to which was added a small quantity of a culture containing *Paramecium caudatum* and *Colpidium* as food. In the case of cultures of *Podophrya* in dilute sea water, the ciliates were also grown in sea water of the same dilution.

Observations were carried out on cultures 5–11 days old. Silk threads carrying the *Podophrya* were mounted on a slide in a drop of fluid from the culture. Except when micromanipulation was required they were covered with a cover-glass; the latter rested on two small pieces of well-washed filter paper which facilitated irrigation. For all observations and experiments the material was irrigated frequently from a pipette with medium from the culture dish to prevent any possibility of a change in the concentration of solutes.

Photographs were taken with a Leica microscope camera. Measurements were made with a screw micrometer eyepiece. A de Fonbrune micromanipulator was used for some experiments. The body volume and surface area of the *Podophrya* were calculated as those of a prolate spheroid from measurements of the major and minor axes.

OBSERVATIONS

Normal feeding

The tentacles of the *Podophrya* normally remained motionless and extended. When a suitable ciliate collided with the *Podophrya*, the tentacles which the ciliate happened to touch at the tip adhered to it. Adhesion of tentacles only occurred with certain ciliates—for instance, *Paramecium caudatum*, *P. aurelia* and *Colpidium* sp., but not *Spirostomum*, *Euplotes* or *Vorticella*. When the prey was caught by one or more tentacles its swimming movements sometimes caused it to rotate and twist the adherent tentacles into a rope-like spiral, and then it often escaped. In other cases its movements merely brought it into contact with the tips of other tentacles, which also adhered. There were no bending movements of tentacles towards the prey.

The process of feeding is illustrated in Pls. 10 and 11. After a few minutes many or all of the cilia of the prey stopped beating. At this time also the body surface of the *Podophrya* in some cases became wrinkled (Pl. 11, figs. B2–5 and C2), although this did not always happen. Some or all of the adherent tentacles then became shorter and stouter, and material from within the prey was seen to flow along these into the *Podophrya*. Granules derived from the prey were often seen to enter the *Podophrya* and to traverse the outer part of the body in sequence as though still in a confined channel. The rate of output of the contractile vacuole of the *Podophrya* increased considerably. The body of the *Podophrya* increased in volume, and during this process any dents or wrinkles which had previously developed in the surface became smoothed out. The prey usually shrank in the region where the tentacles of the *Podophrya* were attached to it. This is illustrated in Pl. 10, in which the anterior end of the *Colpidium* is seen to diminish progressively. The contractile vacuole, visible in figs. A2–A5, provides a fixed point in the *Colpidium* which facilitates a comparison of these photographs with one another. In some cases, particularly in *Paramecium*, a local excavation was formed in the surface in the region where the *Podophrya* was attached (Pl. 11, fig. D).

Paramecium is very much larger than *Podophrya*, and superficially it often appeared little affected when it was finally released by the *Podophrya*. Its contractile vacuoles continued to operate throughout the process of feeding, and in several cases it was seen to swim away some minutes after its release. *Colpidium*, which is much smaller than *Paramecium*, was normally cytolysed. At first its contractile vacuole continued to beat regularly, and damage appeared to be purely local. Later the *Colpidium*, much depleted in size, rounded up. The granules within collected to that side of the spherical remnant to which the tentacles of the *Podophrya* were attached (Pl. 10, figs. A6–8). The rest of the *Colpidium* became much vacuolated. Finally, the contractile vacuole stopped, and there was no sign of life. The passage of material up the tentacles of the *Podophrya* ceased, and the dead *Colpidium* was released.

Stoppage of cilia of prey

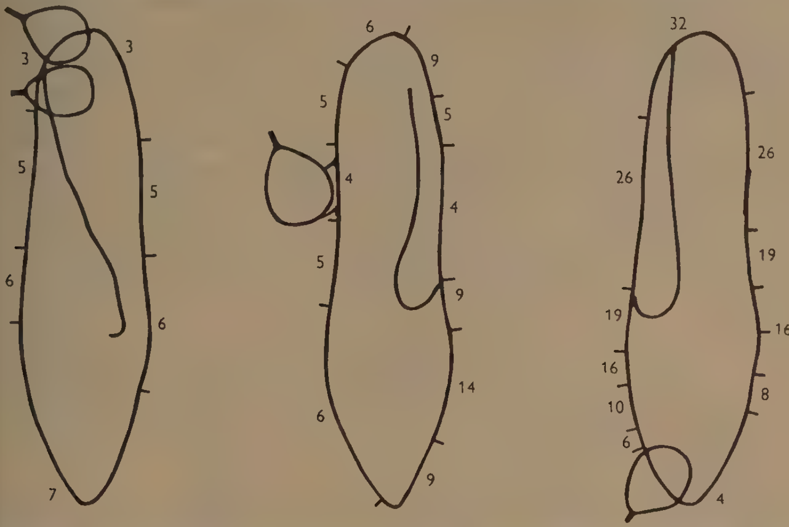
Observations were made of the time after capture at which the cilia in different parts of *Paramecium* stopped. *P. caudatum* and *P. aurelia* were used. Usually the *Paramecium* was caught by its anterior end, and in this case the cilia of the anterior end stopped first. The region of stoppage then extended along the *Paramecium* until it reached the posterior end. In a few cases observations were obtained of *Paramecium* caught by the posterior end. The cilia of the posterior end stopped first, and the area of stoppage extended progressively towards the anterior end. It should be mentioned that, whatever part of the *Paramecium* was held by the *Podophrya*, the tuft of cilia at the tip of the posterior end always appeared to be inactive, and the gullet cilia always continued to beat for much longer than any of the others. Examples of these results are illustrated in Text-fig. 2.

Experiments on wrinkling of pellicle

Wrinkling of the pellicle of *Podophrya* was often observed a few minutes after the *Podophrya* had captured a *Paramecium caudatum*, *P. aurelia* or *Colpidium* sp. The wrinkles occurred in any part of the body surface, and often took the form of a deep cup-shaped depression (Pl. 11, fig. C2). Wrinkling occurred equally strongly in experiments in which the prospective prey had been transferred by centrifuging to some of the culture medium in which the *Podophrya* had been living; this was done to eliminate any possibility that the medium introduced with the prey might be responsible for wrinkling, as, for instance, by an osmotic effect. Wrinkling was never seen to occur in any unfed *Podophrya* adjacent to the one under observation.

Wrinkling of the body surface might be ascribed either to a loss of volume of the body or to an expansion of the body surface or to both. The disappearance of the wrinkles is presumably due to the uptake of material from the prey. It seemed therefore that perhaps on those occasions when there is no wrinkling the uptake of food keeps pace with the process—whatever it may be—by which wrinkling is produced. Accordingly, a comparison was made between the occurrence and persistence of wrinkles in *Podophrya* fed on normal and on partially dehydrated prey. The experiments of the three kinds to be described were suitably interspersed in time.

(*a*) *Podophrya* in tap-water culture were fed on *Colpidium* cultured in tap water. Out of twenty-three experiments strong wrinkling was observed in ten cases, a slight departure from the normal smoothness of outline in seven, and no change in six. Wrinkling, when it occurred, began within 2 or 3 min. and normally the body surface was smooth again in 6–12 min. after the capture of the prey. In two exceptional cases wrinkling persisted until 17 and 40 min. after the capture.



Text-fig. 2. Diagrams showing the time in minutes after capture at which the cilia stopped beating on various parts of the body of the prey.

(*b*) *Podophrya* cultured in 10 % sea water were fed on *Colpidium* which had just been transferred by centrifuging to 10 % sea water. The prey was much shrunk in these experiments. Out of eleven experiments strong wrinkling of the surface of the *Podophrya* occurred in all cases, beginning in from 1 to 7 min. from the time of capture. The wrinkles disappeared after the following times: 38 min., 97 min., 3 hr., $3\frac{1}{2}$ hr., $3\frac{1}{2}$ hr., $4\frac{1}{2}$ hr., $4\frac{1}{2}$ hr. In the remaining four experiments the observations were terminated with wrinkles still remaining after $1\frac{1}{4}$–$3\frac{1}{2}$ hr. In these various experiments no food was seen passing up the tentacles for about the first hour, but later the prey filled out and food particles were seen streaming into the *Podophrya*.

(*c*) *Podophrya* cultured in 10 % sea water were fed on *Colpidium* cultured in or adapted to 10 % sea water. Out of twelve cases wrinkling occurred in six, and the body of the *Podophrya* remained smooth in six. Wrinkling began in from 1 to 4 min., and had disappeared in from 6 to 22 min. after capture of the prey.

Clearly no difference was found between *Podophrya* cultured in tap water when fed on *Colpidium* cultured in tap water and *Podophrya* cultured in 10 % sea water when fed on *Colpidium* cultured in 10 % sea water. However, when *Podophrya* cultured in 10 % sea water was fed on *Colpidium* only just transferred to 10 % sea water the wrinkling was universal and much prolonged, and the uptake of food was greatly postponed. This conclusion is supported by observations of *Podophrya* feeding on *Paramecium.*

The use of partially dehydrated prey provided an opportunity for studying the phenomenon of wrinkling without the complications due to the subsequent uptake of food. It was already known that an unfed *Podophrya* is only capable of a quite limited swelling by osmosis, unless it happens already to be wrinkled, although it can be caused to shrink greatly by a hypertonic solution (Kitching, 1951). This suggested that an expansion of the body surface of pellicle might be important in permitting the great swelling which occurs during feeding. Experiments were therefore carried out to determine the change in distensibility of the body which takes place during the wrinkling process. A comparison was made between the capacity for osmotic swelling on transfer from 10 % sea water to tap water of unfed *Podophrya* and of *Podophrya* which had been fed on partially dehydrated prey (as described above) but from which the prey had been removed with a microneedle when wrinkling had developed. Because some *Podophrya* cultured in 10 % sea water were found in any case to be wrinkled, unwrinkled *Podophrya* were carefully selected. The results are shown in Table 1. The increase in surface area was much greater in those *Podophrya* in which wrinkling had been induced by the capture of prey. Agitation of an unfed *Podophrya* with a microneedle to an extent consider-

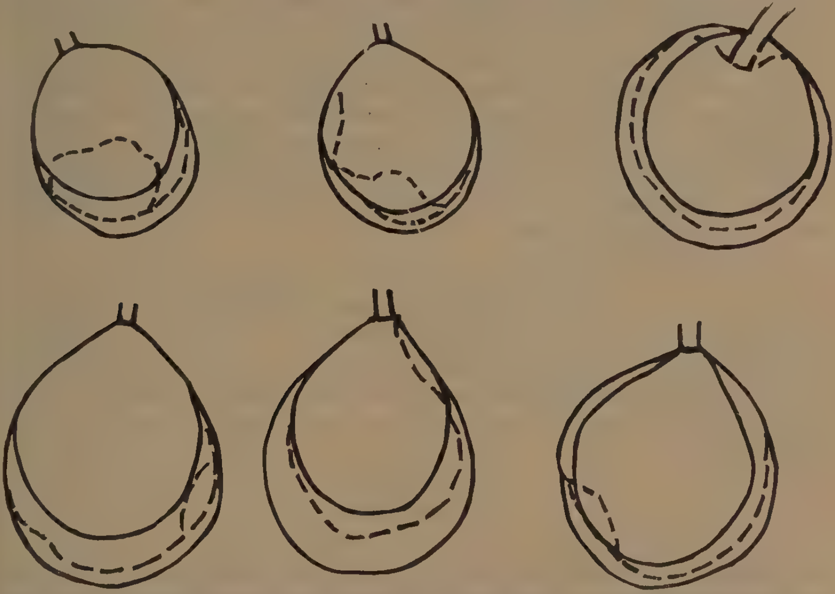
Table 1. *The effect of previous feeding and removal of food on the increase in surface area of the body when* Podophrya *cultured in* 10 % *sea water were transferred to Bristol tap water*

Date	Experiments in which prey was captured but was removed when the surface of the *Podophrya* had become wrinkled		Experiments in which no prey was captured	
	Surface area (μ^2) in 10 % sea water	Percentage increase in surface area after transfer to tap water	Surface area (μ^2) in 10 % sea water	Percentage increase in surface area after transfer to tap water
18. v. 50	2485	37	2870	3
8. v. 50	3190	33	2940	2
8. v. 50	2240	45	3800	16
8. v. 50	—	—	1535	3
8. v. 50	—	—	2650	6
8. v. 50	—	—	3240	3
15. v. 50	2990	30	2165	2
26. i. 51	—	—	5320	3
6. ii. 51	3810	26	—	—
7. ii. 51	5480	16	3200	1
15. ii. 51	4320	34	4070	5
15. ii. 51	3430	20	3490	9
26. ii. 51	4640	35	5500	17
26. ii. 51	3470	21	3170	12
26. ii. 51	3580	38	3830*	9
26. ii. 51	—	—	2990*	4

* Tickled with microneedle before transfer to tap water.

ably greater than that normally caused by prey did not produce any wrinkling or increase in surface area.

Finally, the process of wrinkling was studied by a comparison of photographs taken at intervals of a few minutes or less during the early stages of feeding. This was done in the hope of determining whether any diminution of body volume is associated with wrinkling, and in order to compare the size of the body when the wrinkles were just disappearing with the original size; this comparison would indicate any increase in surface area due to wrinkling. Examples of the photographs are shown in Pl. 11, and tracings obtained by projection from negatives are given in Text-fig. 3.



Text-fig. 3. Outlines projected from photographs of *Podophrya* in the process of feeding. Continuous inner line, immediately after capture of prey; broken line, at the time of greatest wrinkling; continuous outer line, at the point of disappearance of wrinkles. The three drawings in the top row are of *Podophrya* cultured in tap water and feeding on prey cultured in tap water. The three drawings in the bottom row are of *Podophrya* cultured in 5 % sea water and fed on prey recently transferred to 5 % sea water. The *Podophrya* was in these cases transferred to tap water after wrinkling to accelerate swelling.

The photographs shown in Pl. 11, figs. B 1–5 and C 1–3, are of normal *Podophrya* cultured in tap water and fed on normal prey. In series B two *Podophrya* are attached to one *Colpidium*. One of these shows strong wrinkling, which in this case persisted until the prey was practically consumed. The other *Podophrya* did not wrinkle, but appears to have obtained most of the food. It is clear from the photographs that the wrinkling involved an increase in the area of the body surface. It should be mentioned that wrinkling was in no way confined to cases in which two *Podo-*

phrya were attached to the same prey. It was frequently seen when the prey was not shared. This is illustrated in series C. Here the wrinkling merely took the form of a small cup-shaped depression, which soon filled up. In view of the increase in the surface during wrinkling it is not possible to decide whether or not a change of volume also occurred. The small volume of a depression such as that shown in C 2 or seen in early stages of wrinkling in other cases would be compensated by an insignificantly small extension of the overall outline of the body. On the other hand, in all cases in which wrinkling became strongly marked there was also a considerable extension of the outlined area. This is illustrated in Text-fig. 3, in which the outline is given for *Podophrya* (*a*) immediately after capture of the prey, (*b*) at the time of greatest wrinkling, (*c*) when the wrinkles were on the point of disappearance. The third condition was obtained either in the normal course of feeding, or (for some *Podophrya* in 5 % sea water) by osmotic swelling. In either case the outlines show that at the moment of disappearance of the wrinkles the body was much larger than it had been originally. They confirm that the surface of the body expands during wrinkling.

Rate of increase of body volume during feeding

Observations were made of the rate of increase in body volume of *Podophrya* during feeding. The results are shown in Text-fig. 4. Points are plotted only for those measurements taken when the body surface was smooth in outline. The number of tentacles attached to the prey is also given in each case, although it must be realized that the uptake of food does not necessarily occur through all the tentacles attached.

DISCUSSION

External digestion

The observations of Iziumov (1947), already outlined, strongly suggest that some digestive action takes place within the prey itself. The progressive stoppage of cilia from the region of attachment of the *Podophrya* outwards, and the local destruction of the prey in this region, fit in with this hypothesis, although other explanations are possible. However, this digestive action must be very localized. For instance, it does not normally stop the contractile vacuole of the prey until late in the feeding process.

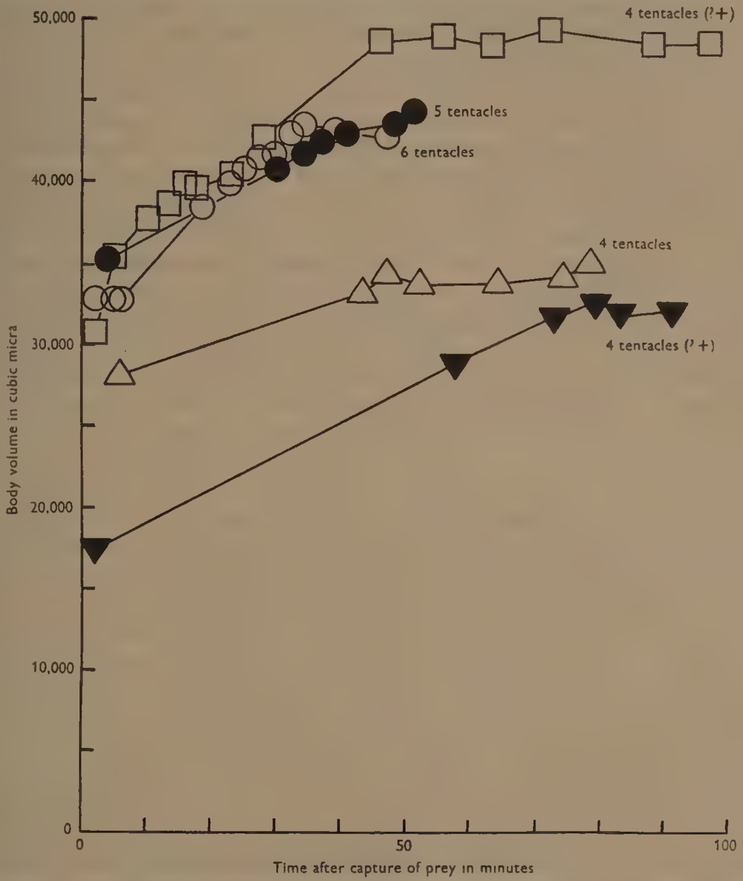
External digestion implies that digestive material or juice, however small in quantity, must pass from the *Podophrya* into the prey. There is as yet no direct evidence for this. Although it might be tempting to ascribe the wrinkling of the *Podophrya*, which often follows soon after the capture of prey, to an injection of digestive juice into the prey, the evidence presented does not warrant any such supposition. I cannot either confirm or deny this possibility. Actually the quantity of material might be exceedingly small; sufficient might be present at the tips of the tentacles.

Mechanism of suction

The wrinkling of the body surface of *Podophrya* which often occurs within a few minutes of capture of prey is due to an expansion or growth of the body surface.

At the least this expansion enables the *Podophrya* to accommodate a considerable volume of food. The expansion no doubt occurs even when no wrinkles are seen, wrinkling being prevented by the simultaneous uptake of food.

Does expansion of the body surface merely permit the uptake of food by the action of some other force, or does it represent a mechanism of active suction? The difference of pressure necessary to drive liquid food material along the tentacles of the *Podophrya* can be estimated very roughly, on certain not unreasonable assumptions, by the use of Poiseuille's formula. The rate of swelling of the *Podophrya* cannot be regarded as an accurate measure of the rate of uptake of food, since it is possible (Kitching, 1938, p. 414) that water derived from the food is removed by the extra activity of the contractile vacuole which is associated with feeding.



Text-fig. 4. The rate of swelling of the body of *Podophrya* during feeding. Points have been plotted only for those readings obtained when the outline was smooth and free from wrinkles.

Rudzinska & Chambers (1951) have estimated that in the suctorian *Tokophrya infusionum* this increased activity accounts for a very large proportion of the liquid ingested. In *Podophrya* (Kitching, unpublished data) the extra activity amounts usually to 3–6 μ^3/sec., or, say, 20,000 μ^3/hr. The rate of swelling of the body may also be estimated as not more than 20,000 μ^3/hr. The viscosity of the material flowing along the tentacles is not known and is difficult to estimate. If we grant the claim of Fetter (1926) that the viscosity of the endoplasm of *Paramecium* is about 8000 times that of water, it is necessary to assume a digestion or structural breakdown before this material could pass along the tentacles. It must be remembered that with a material as complex as protoplasm the 'viscosity' is likely to depend on the method and conditions of measurement. On the assumption that the viscosity of this material under the conditions in question is twice that of water, that the length of the tentacles is 25 μ, that the internal diameter of the inner tube is between $\frac{1}{2}$ and 1 μ, and that three tentacles are functional, a difference of pressure between about 1 and 0·1 cm. water would be required. This estimate is exceedingly rough, and would be seriously affected by any error in the estimate of the internal diameter of the inner tube of the tentacles, which is in any case only a guess. The hydrostatic pressure within the ciliate *Spirostomum ambiguum* has been estimated by Picken (1936) as 3–7 cm. of water, although this value must also be regarded as very rough. It seems very possible, provided that any structural viscosity of the protoplasm consumed is destroyed, that the hydrostatic pressure of the prey might be sufficient to account for the passage of material along the tentacles of the *Podophrya*. It is assumed that there is little if any positive pressure within the latter. However, the rate of uptake of food does not diminish during the later stages of feeding, so that it would be necessary to assume that the tone of the body surface of the prey was maintained. A more serious objection is the observation, illustrated in Collin's monograph (Collin, 1912), that *Choanophrya infundibulifera* can draw in particles of food from a distance. Only suction could account for this. If suction is admitted in one case, it is difficult to reject it in other cases, for which it would clearly provide the most reliable mechanism of feeding. However, the evidence presented in this paper does not provide any information as to whether a negative pressure is in fact achieved in *Podophrya*. Suction might well be effected by an expansion of the body surface, so long as there is some resistance to a collapse of the body wall inwards. Wrinkling of the surface would then represent a failure of that resistance to prevent local inward collapse, due perhaps to delay in liquefaction of the protoplasm of the prey at the point of attachment of the tentacles. The necessary resistance to inward collapse might be provided by a gelated layer underneath the plasma membrane of the *Podophrya*. Another interesting problem is set by the expansion of the cuticle which must also take place. Growth of the body surface presumably also occurs during the process of internal budding which occurs in this species.

SUMMARY

1. *Podophrya* sp., studied in this work, feeds on various holotrich ciliates. The tips of the tentacles adhere to the prey. Within a few minutes the cilia of the prey in the region where the *Podophrya* is attached stop beating. The stoppage spreads from here outwards over the surface of the prey. In some cases the surface of the *Podophrya* becomes wrinkled. The prey is broken down locally in the region of attachment, material from the prey flows up the tentacles into the *Podophrya*, and the *Podophrya* gets bigger, any wrinkles that may have formed becoming smoothed out in the process.

2. Wrinkling of the *Podophrya* is shown by observations and experiments to be due to an expansion or growth of the body surface. This expansion serves to reduce the hydrostatic pressure within the *Podophrya*, so that feeding may proceed.

3. It is suggested that expansion of the body surface, coupled with a supposed resistance to inward collapse, might provide suction for feeding. Wrinkling of the surface is ascribed to a local collapse which may be supposed to occur when uptake of food material from the prey fails to keep pace with the expansion.

I am indebted to Prof. J. E. Harris for valuable suggestions and criticisms, and to Mr E. Livingstone for maintaining a steady supply of excellent cultures.

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EXPLANATION OF PLATES

PLATE 10

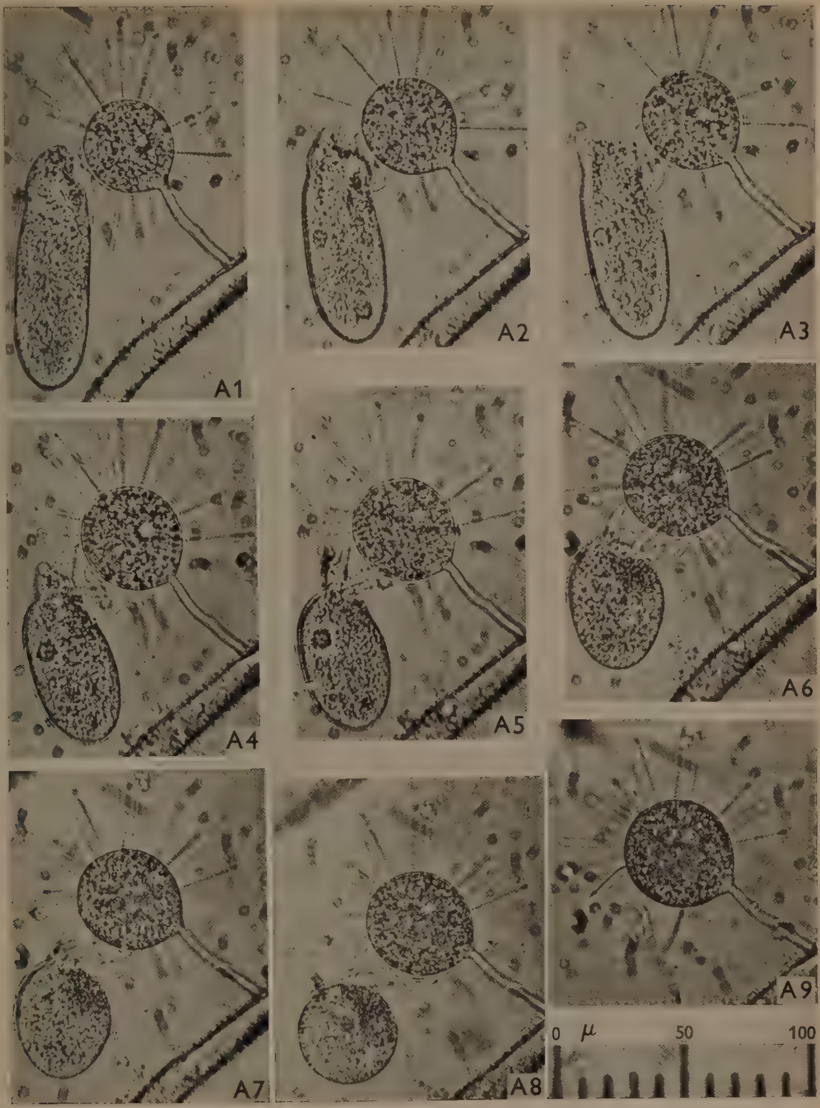
Series A. *Podophrya* feeding on *Colpidium* sp. Both were cultured in Bristol tap-water medium and were observed in some of the medium from the *Podophrya* culture. The photographs were taken at the following times after capture of the prey: A1, 2 min.; A2, 9 min.; A3, 10 min.; A4, 18 min.; A5, 23 min.; A6, 38 min.; A7, 44 min.; A8, 61 min.; A9, 102 min. No wrinkling of the body surface of the *Podophrya* occurred on this occasion. This series illustrates the disintegration of the prey in the region of attachment of the tentacles of the *Podophrya*. The contractile vacuole of the *Colpidium*, visible in A2–5, provides a fixed spot for comparison. Granules are seen to collect on the side of the *Colpidium* towards the *Podophrya* in A6–8. The *Colpidium* has been reduced to a spherical remnant in A8. It has been released in A9.

PLATE 11

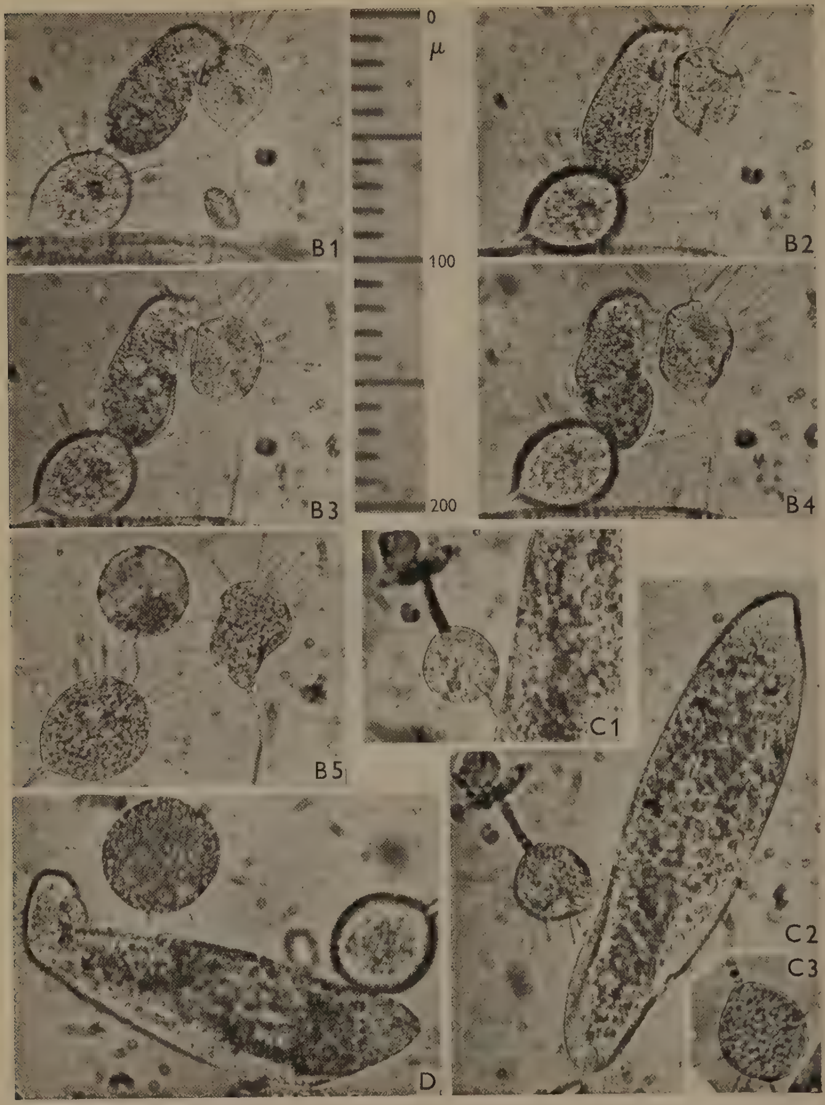
Series B. Two specimens of *Podophrya* feeding on a single *Colpidium* sp. All were cultured in Bristol tap-water medium and observed in some of the medium from the *Podophrya* culture. The photographs were taken at the following times after capture of the prey: B1, 2 min.; B2, 5 min.; B3, 6 min.; B4, 8 min.; B5, 39 min. The right-hand *Podophrya* shows a progressive wrinkling and expansion of the body surface, whereas the left hand *Podophrya* increases in size without becoming wrinkled.

Series C. A single *Podophrya* feeding on a *Paramecium caudatum*. The photographs were taken at the following times after capture of the prey: C1, ¾ min.; C2, 5 min.; C3, 36 min. A dent in the body surface of the *Podophrya* is seen in C2.

Photograph D. Two specimens of *Podophrya* feeding on a *Paramecium caudatum*. The left hand *Podophrya* has nearly finished feeding, and the *Paramecium* is excavated in the region of attachment of the tentacles.



KITCHING—OBSERVATIONS ON THE MECHANISM OF FEEDING IN THE SUCTORIAN *PODOPHRYA*



KITCHING—OBSERVATIONS ON THE MECHANISM OF FEEDING IN THE SUCTORIAN *PODOPHRYA*

THE CO-ORDINATION OF INSECT MOVEMENTS

I. THE WALKING MOVEMENTS OF INSECTS

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(*Received* 23 *October* 1951)

(With Plate 12 and Eight Text-figures)

Though walking forms part of many insect behaviour patterns, the mechanism of insect walking has received relatively little attention in recent years. As a preliminary to a later investigation of neuromuscular co-ordination in insects, the normal pattern of walking movements in the cockroach and other insects has been studied. The results obtained and an account of the functional anatomy of the insect leg are given in this paper. The forces involved have not been measured, but a preliminary analysis of the mechanics of walking has been made with the help of 'stills' from cinematographic films. The use of cinematography greatly facilitates such an investigation.

Borelli (1680), in his *De motu animalium*, did not give a true picture of insect leg movements, but his suggestion concerning their order of protraction has been substantiated in the present research. In reviewing Borelli's account, Demoor (1890) was very critical of the supposition that it would be advantageous if the three ipsilateral legs of a hexapod were lifted in the order 'initium motus fieret a posteriori, cui succederet medius et ultimo anticus'. This suggestion was based not on observation but on the theoretical principle that the hindleg should move first in order to propel the centre of gravity forwards before the other legs were lifted. Demoor maintained that the foreleg of one side is lifted simultaneously with the middle leg of the opposite side while the ipsilateral hindleg is raised a little later, but all three legs of the triangle are placed on the ground instantaneously.

Paul Bert (1866) considered that the legs of *Carabus* are lifted in the order*: L3 and R2; R1; L2 and R3; L1 and R2; etc. This rhythm agrees with the principle of Borelli, but a study of films has shown that there is a short but definite delay between the lifting of the middle and contralateral hindleg. Bert was one of the few observers to recognize that insects have more than three legs on the ground during many phases of the locomotory cycle. Lloyd Morgan (1886) also drew attention to this fact when he wrote 'Much has been written on "the horse in motion". Can any readers of *Nature* supply me with references to published matter on the subject of hexapod progression?' In a reply, Wilkins (1887) stated: 'In general I found that the mode of projection in articulates does not differ essentially from what we see in vertebrates.' This attitude resulted from the opinion then held by the majority of workers (E. Weiss, Müller, Bürmeister, Graber, Dahl, Carlet, etc.) that insect movement is accomplished by the alternation of two tripods of support, each composed of the foreleg and hindleg on one side, together with the middle leg of the opposite side. Thus, although having six legs the insect was envisaged as moving them in two alternating groups just as in a biped. Demoor extended this concept to include the majority of Arthropods, as he thought that they also used two alternating groups of diagonal legs. He concluded that the movement of insects, arachnids, and probably Crustacea, too, together with man and tetrapods, conforms to the general description that 'La marche est une séries de chûtes successivement arrêtées'.

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* The convention adopted in distinguishing the different legs is as follows: R and L denote the right and left legs of each segment which are numbered from in front. Thus L2 is the left middle leg.

Cinematography discloses that many of these conclusions concerning the rhythm of leg movements in arthropods are oversimplified. The concept that movement is a series of checked falls seems to have little meaning except in the case of bipeds or tetrapods which are moving fast and thereby possess dynamic stability. The walking tetrapod may stop at any phase of the cycle and not fall over, as the centre of gravity always lies within the area of support. Paul Bert appears to have been the only worker to have denied this concept when applied to insects, an attitude which followed from his observation that there are often more than three legs on the ground at any one time.

There has been general agreement with regard to the roles fulfilled by the three pairs of legs during walking. The forelegs were considered to have a tractive function, and the hind pair to push while the middle legs act as fulcra. During the present century, the focus of research has moved from the mechanism of movement to a study of the role of the brain and different parts of the nervous system in the co-ordination of locomotory movements. Baldi (1921, 1922, 1924, 1938) made an extensive study of this aspect and also included an account of the leg movements of *Periplaneta* with which the following account is in substantial agreement. Baldi laid great stress on the lack of co-ordination in the movements which brought about any progression that followed removal of the suboesophageal ganglia. During the course of work on the importance of the higher centres in the locomotion of *Mantis*, Roeder (1937) made the interesting observation that the rhythm of leg movements might vary under different conditions. When the insect is quietly walking the order is L3, L2, R3, R2; when more excited the rhythm is L2–R2, L3–R3; when climbing L3–R1, L2–R3, L1–R2. Apart from this observation there has been no suggestion that the limb order might vary at different speeds, although Pringle (1939) considered that a distinction between running and walking was justifiable in the cockroach.

MATERIALS AND METHODS

Detailed studies were made on the cockroaches, *Periplaneta americana* and *Blatta orientalis*, and various beetles, *Dytiscus marginalis*, *Hydrophilus piceus*, *Carabus violaceus*, *Chrysomela orichalcea* and *Blaps mucronata*. The walking movements of other insects did not appear to differ from these in any essential feature. The insects were usually filmed from above as they walked over graph paper, but simultaneous side and ventral views, taken with the aid of a mirror, proved most valuable in describing the cycle of individual leg movements.

A Sinclair ciné camera was used at speeds of 16–32 frames per sec. with illumination provided by two Photoflood lights at a distance of about 3 ft. This lighting disturbed the cockroaches, but their negative phototaxis assisted the photography as they ran towards a dark box placed at one side of the field. It was more difficult to obtain films of the insects moving at the slower speeds because of this disturbing effect, but fortunately this is the range over which the eye can follow the movements most easily.

RESULTS

I. *Morphological*

Before considering the morphology and movement of the legs it is necessary to define the terms to be used in this account.

Protraction is the complete movement forwards of the whole limb relative to its articulation with the body.

Retraction, similarly, describes the remaining half of the cycle between the instant when the leg is placed on the ground and the time when it is raised and protraction commences.

Promotion and *Remotion* refer to the corresponding anterior and posterior movements of the coxa with respect to the body.

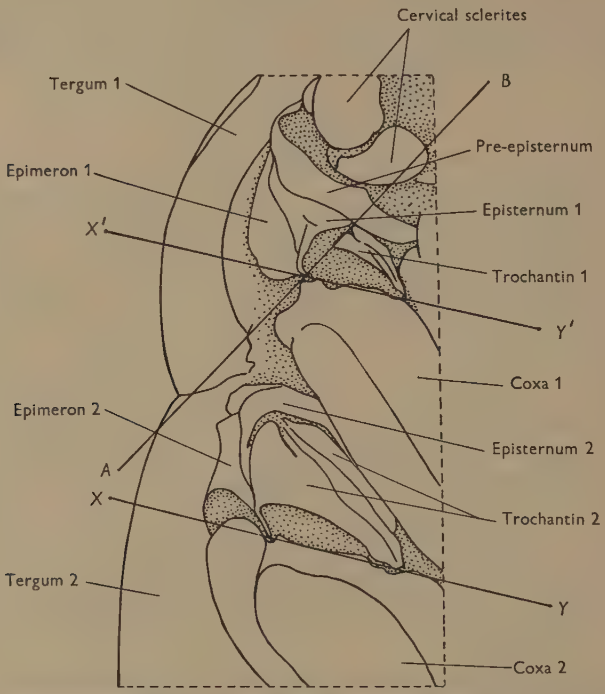
Adduction and *Abduction* are likewise movements of the coxa brought about by extrinsic muscles. The former is a movement towards and the latter away from the body.

Levation and *Depression* describe parts of the protractor and retractor movements in which the leg is raised or lowered relative to the ground.

Extension and *Flexion* express the relationship between two adjacent segments of the legs; flexion occurring when the angle between them is reduced and extension when this is increased.

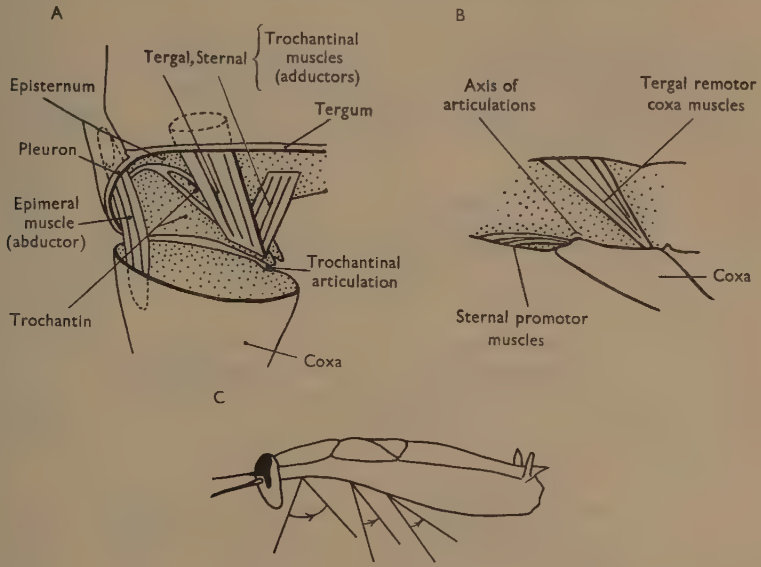
Cockroaches inhabit secluded crevices and show many modifications in relation to their mode of life. This is particularly evident in the structure and functioning of the thorax, and consequently an account of the locomotion of *Blatta* cannot be generalized for all insects. The leg articulations are placed more ventrally than in most insects, due to the extensive desclerotization of the sternum. These articulations are dicondylic, one episternal and the other trochantinal, in all the legs, and they limit the range of movements very closely. The axis of these articulations is inclined forwards at an angle of 60° to the ventral axis; a feature which enables the leg to obtain a more anterior *point d'appui* for any given degree of promotion than would be possible if the axis was at right angles to the mid-ventral line. The movement of the mesothoracic and metathoracic coxae is almost entirely restricted to the antero-posterior plane determined by this joint. A limited amount of adduction and abduction can take place, however, because of the flexibility of the ventral exoskeleton.

The range of movement of the prothoracic pair is much greater, and this is related to the nature of the coxal articulations. There are again episternal and trochantinal condyles which partially restrict the movement of the coxa to their axis. The greater mobility of the foreleg is to be attributed to the smaller size of the trochantin which is not wedged into the episternum and can move upon it (Text-fig. 1). Hence the axis of this joint is not so rigidly fixed as in the mesothoracic and metathoracic legs, because the trochantinal articulation can move relative to the episternal condyle.



Text-fig. 1. *Periplaneta.* Ventral view of the coxal articulations of the right fore and middle legs. Promotor-remotor movements of the mesothoracic coxa can only occur about the axis $X-Y$ determined by the episternal and trochantinal condyles. In addition to the axis $X'-Y'$ of similar condyles, the front coxa can also move about the axis $A-B$ as the trochantin is not wedged so firmly into the episternum.

Movement of the coxa is brought about by extrinsic muscles having their origin in the thorax and insertion on the proximal end of the coxa. Carbonell (1947) has given a detailed account of the many muscles involved, but for the present purpose it is more important to recognize the relationships of the main functional groups, and these are shown in Text-fig. 2. The remotors are the most powerful of these, particularly the tergal remotor coxa muscle which is inserted on the posterior rim of the coxa. Adduction is accomplished by muscles with their origin on the tergum lateral to that of the remotor, and their insertion on the inner edge of the trochantin near the coxo-trochantinal articulation. Abduction is chiefly brought about by the elasticity of the sclerites, but it is aided by the activity of a muscle originating on the epimeron and subalar plate, and inserted on the meron, i.e. lateral to the coxo-episternal condyle. The promotors have their origin on the anterior edge of the episternum and are attached to the anterior coxal rim. In the prothoracic legs, however, the main promotor coxa muscle is absent and its function fulfilled by a muscle inserted near the coxo-episternal articulation and with its origin on the anterior part of the prothoracic shield. It is due to the upward component of this muscle's activity that a 'buckling' of the episternal-trochantinal hinge takes place during promotion.

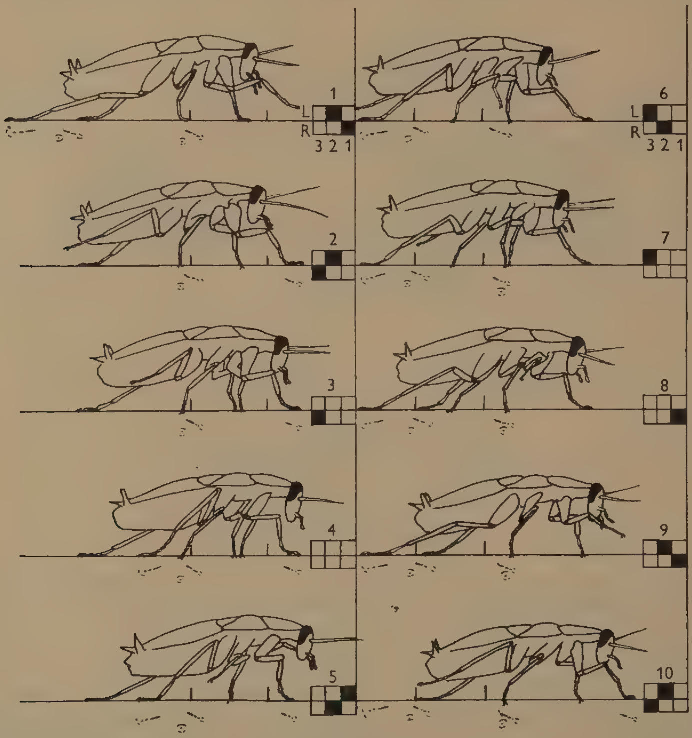

A
Tergal, Sternal
Trochantinal muscles (adductors)
Episternum
Tergum
Pleuron
Epimeral muscle (abductor)
Trochantinal articulation
Trochantin
Coxa
B
Axis of articulations
Tergal remotor coxa muscles
Coxa
Sternal promotor muscles
C

Text-fig. 2. A, diagram showing the position of the main adductor and abductor muscle groups of the hind coxa seen from above and behind; B, diagram of the promotor-remotor mechanism of the hind coxa in side view; C, diagram indicating the range of angular movements of the three coxae during normal walking of the cockroach.

Pringle (1938) has given an account of the functional morphology of the joints and the intrinsic musculature of the legs themselves which are typical of a running insect. The orientation of these appendages with respect to the body is not normal, however, as the middle and hind pairs always point obliquely backwards (Text-fig. 2C), so that the extensor trochanteris muscles play a predominant role in the propulsive strokes of these legs.

II. *The cycle of individual leg movements*

The movements of the three pairs of legs were determined from simultaneous ventral and lateral photographs obtained by means of an inclined mirror. A series of diagrams was traced from these, and it shows the movements of the different limb segments throughout one complete cycle of walking (Text-fig. 3). The markings beneath each diagram are the footprints obtained when the insect was allowed to walk over smoked paper, a technique employed by many previous investigators.



Text-fig. 3. *Blatta.* A series of drawings taken from successive frames of a film (30 frames/sec.) to show the sequence of leg movements in side view during normal walking. The legs which are off the ground during each phase are indicated by the black squares on the right of each drawing and the markings left by the insect when walking over smoked paper are shown beneath.

In the fully protracted position of the foreleg, maximal promotion of the coxa is obtained by means of the episternal-trochantinal hinge. All the joints of the leg are extended so that the new *point d'appui* is in front of, and lateral to the head. During retraction the axis of the limb is moved backwards relative to the body, so that the angle of inclination (α) of this axis gradually decreases. There is also a decrease in the angle (θ) between the limb and transverse body axes as seen in ventral view (Text-fig. 4A) The activity of the remotor coxae, flexor trochanteris, and flexor tibia muscles produces these movements which draw the body forwards and towards the side of the active limb. When the limb axis has passed the vertical position, an extensor thrust is exerted along the axis by the contraction of the extensor trochanteris and extensor tibia muscles.

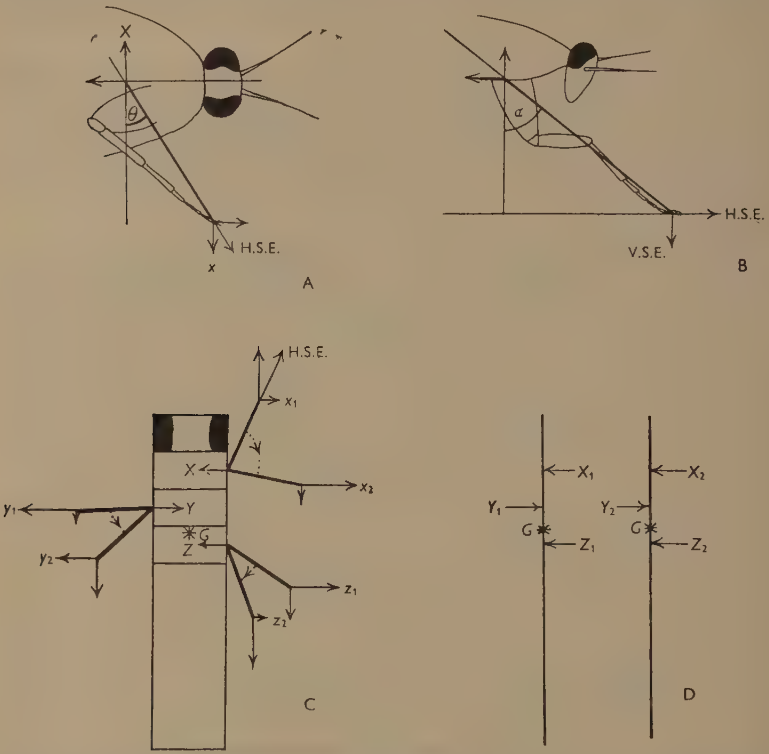
Protraction commences when the limb is levated from the ground as a result of the activity of the flexor trochanteris and flexor tibia muscles once again. Contraction of the promotor coxae muscles now swings the whole leg forwards and outwards while it extends as the result of increased activity by the extensor trochanteris and extensor tibia. Retraction commences the cycle once again as soon as the leg has obtained a new *point d'appui*.

The intrinsic leg muscles thus undergo four phases in the cycle, two of extension and two of flexion. The extrinsic muscles undergo one of promotion and one of remotion. When the leg is placed on the ground and starts to retract, the horizontal strut effect (Barclay, 1946) will tend to retard forward movement of the body, but, as a result of the activity of the remotor muscles, the horizontal lever effect is in the opposite direction and propels the body forwards. When the angle of inclination (α) begins to increase again, after its reduction to zero during the earlier stage of retraction, the intrinsic muscles exert an extensor thrust along the axis of the limb. From Barclay's work on the patterns of extrinsic muscle-couples in the locomotion of toads, we should expect the promotor coxae muscles to be active during this latter phase of the cycle so that the horizontal strut and lever effects at the feet are again in opposite directions. If this is so, it can be seen that the change-over from retraction to protraction will be a smooth one because the contraction of the promotor muscles, which follows levation at the commencement of protraction, will have already begun at the end of retraction. This example serves to emphasize the impossibility of describing adequately any movement as a succession of discrete events, because the muscles contract and relax in a complex but more or less regular sequence of activities which grade one into another.

The coxae of the second pair never become perpendicular to the body during walking and hence the strut effect always assists forward movement. Protraction of these legs consists of promotion of the coxa, flexion of the trochanter and slight flexion of the tibia on the femur. The new *point d'appui* is obtained as the joints begin to extend, and at this instant the limb axis is at right angles to the body axis when viewed from above. Remotion of the coxa gives a lever action for a short time but the main propelling force on the body is produced by extension of the trochanter, and to a lesser extent of the tibia, towards the completion of retraction.

The promotor and remotor movements of the metathoracic coxae are even less than those of the middle pair of legs, and once again the extensor trochanteris and extensor tibia muscles are mainly responsible for the propulsive action of the legs.

Evidently the legs of the cockroach can function as levers and inclined struts. The former activity being of greatest importance in the forelegs while the hindlegs are functioning almost exclusively as inclined struts. This gradation is related to differences in the coxal articulations and musculature affecting the range of angular movements of the coxae (Text-fig. 2C). This diagram was obtained from photographs of *Blatta* walking over glass and under such conditions of low friction it is interesting to notice that the legs were held a little more vertical than on rougher substrates.



Text-fig. 4. Diagrams indicating the forces operating when the insect legs are functioning as inclined struts. A, ventral view; B, side view to show the angle of inclination (α) of the fore limb axis. The force at the tarsi may be resolved into horizontal (H.S.E.) and vertical (V.S.E.) strut effects. C and D, diagrams to show the changes in the relative magnitudes of the lateral components (x, y and z) of the horizontal strut effects at the *points d'appui* of the foreleg, middle and hindlegs during the retraction of a triangle of support. Equal and opposite to these components are the forces exerted on the body (X, Y and Z) which will produce turning-couples on the body. It can be seen that: $x_1 < x_2$, $y_1 > y_2$, $z_1 > z_2$ and hence $X_1 < X_2$, $Y_1 > Y_2$ and $Z_1 > Z_2$. The body axis will therefore tend to rotate in an anticlockwise direction during the retraction of this triangle of support.

Only the longitudinal components of the forces acting at the feet have been considered so far because these are concerned in propelling the body forwards.

The transverse components (x, y, z) are important, however, in determining the lateral displacement of the body (Text-fig. 4). Equal and opposite to these reactions at the feet are the transverse forces (X, Y, Z) operating on the body, and these will produce turning couples which must balance one another if movement is to be rectilinear. This is very nearly so because the path followed by a walking cockroach is almost a straight line. There is a slight deviation, however, as the body axis rotates a little towards the side on which a foreleg is being placed on the ground and then it gradually rotates in the opposite direction during retraction of this leg. A simple hypothesis can be constructed to account for this observation.

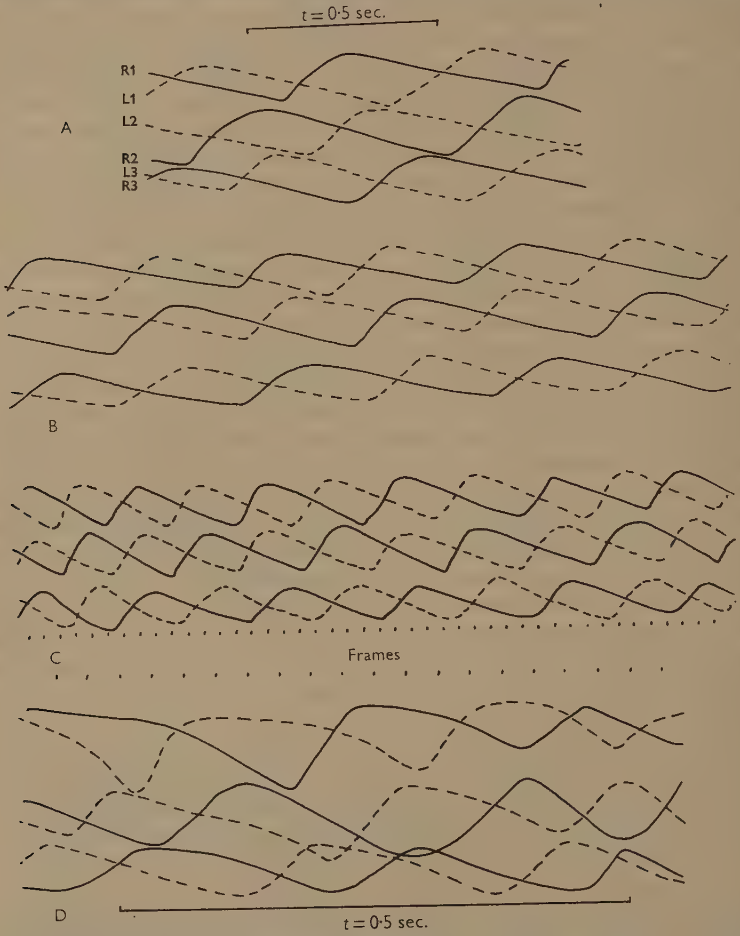
If the legs are considered to function as rigid struts along their limb axes, the horizontal strut effects at the tarsi may be resolved into longitudinal and transverse (x, y, z) components. The axial thrust of a limb when functioning as an inclined strut will exert a force on the body which will likewise have transverse (X, Y, Z) and longitudinal components equal and opposite to those at the tarsi. There will be a change in the relative magnitudes of these components during the retraction of the three legs of a triangle which, for the present purpose, may be assumed to retract simultaneously. As the perpendicular distance of the articulations from the centre of gravity (G) remains constant, such changes in the transverse components will determine the pattern of turning couples to which the body is subjected during progression. It can be seen from the geometrical relationships that the transverse component (x) of the foreleg increases while that of the mesothoracic (y) and metathoracic (z) legs decreases during the retraction of a given triangle (Text-fig. 4C). Such a pattern of couples will result in the observed rotation of the body axis away from the foreleg which is on the ground. When the other foreleg is placed on the ground it draws the anterior part of the body towards that side for a short time and then its strut effect brings about a contralateral movement as described above.

This hypothesis is an oversimplification for several obvious reasons. In the first place, the legs are not rigid struts, but can vary their axial thrusts by means of the intrinsic musculature. Secondly, the lever action of the legs which involves the extrinsic muscles has been neglected. And finally the three legs of a triangle are not retracted synchronously but in the order R1, L2, R3, L1, R2, L3, R1. A consequence of this rhythm is that the middle leg will be placed on the ground and so exert its maximum turning moment on the body at an instant when the transverse component (X) due to the first leg is increasing. Similarly, the component Z will be maximal when X is relatively large. It is apparent, therefore, that these factors will tend to reduce the net turning-moment on the body throughout the whole walking cycle.

Such considerations suggest that the delay between the instant at which the three legs of a tripod are protracted may have functional significance in that it will decrease the oscillations of the body along the direction of movement. It is certainly true that movement is much more rectilinear in insects, e.g. *Chrysomela*, in which the legs move at very nearly equal intervals after one another than those such as *Blaps*, in which the movement of the members of a tripod are only separated by very short intervals.

III. *The rhythm of walking movements*

The antero-posterior movements of the legs relative to the head can be plotted out from the films. The graphs obtained (Text-fig. 5) show the time relations of the protraction and retraction phases for each leg and the rhythm of the six legs relative to one another. The predominant order of protraction is R1, L2, R3, L1, R2, L3 (Pl. 12), in which the first three legs and the second three are protracted very soon after one another but not simultaneously. This delay between the protraction of the three legs of a triangle is true at all velocities, although it is only apparent at the



Text-fig. 5. Graphs showing the movements of the legs relative to the head during normal walking of A, *Chrysomela*; B and C, *Blatta* at two different speeds; D, *Dytiscus*. Legs of the right side are shown by continuous lines and the dotted lines indicate the movement of the legs on the left side.

faster ones if the camera speed is increased or flash cinematography employed. The same rhythm is present in films of *Chrysomela*, *Dytiscus* (Text-fig. 5), *Hydrophilus* and *Blaps*. The former beetle has a more regular distribution of the protractor phases of the different legs, but even here the distinction into two tripods is fairly definite (Text-fig. 5A). Films also showed that *Carabus* uses this rhythm and that it moves along an almost straight line. The usual diagram (adapted from von Lengerken) given to illustrate insect walking is somewhat misleading, therefore, as it suggests that the path followed by *Carabus* is a very zig-zag one.

At the beginning of this paper it was indicated that the majority of workers have analysed the rhythm of insect and other arthropodan movements into two groups of legs moving alternately. Von Holst (1935) maintained that the following two rules are obeyed by nearly all insects, not only the normal ones but also those from which legs had been removed: (*a*) adjacent legs of a side alternate; (*b*) diagonal legs work synchronously. Analysis of films has shown that these rules are not strictly correct. The first is true in principle, but a more important relationship is that the wave of protraction passes forwards along the legs of each side, and in insects no leg is protracted until the one behind has taken up its supporting position. The second rule is plainly false, at least for insects and arachnids, and probably a closer investigation will confirm this in other arthropods.

It is generally possible to distinguish two groups of legs in which the three legs move very soon after one another, but this is not always the case. If a rigid alternation of tripods was employed, only three legs should be supporting the body at any one time during the locomotory cycle. But Text-fig. 3 clearly shows a complete cycle during which there are never less than four legs on the ground at any one instant. In fact, there is one phase (4) when all six legs are supporting the body. Furthermore, the legs of *Chrysomela* are often retracted at almost equal intervals of time after one another, and the distinction into tripods is not so evident, thus: L3–3.4–R1–2.7–L2–2.8–R3–3.2–L1–3.1–R2–3.2–L3, etc. (the figures indicate the average number of frames which separate the protraction of the legs concerned).

Additional evidence is provided by the observation of rhythms which cannot be analysed into such a system of alternating tripods, and these indicate the possible basic units which make up the normal walking rhythm. The drawings in Text-fig. 6 show a cockroach in which the legs are protracted in the rhythm R3, R2, R1, L3, L2, L1, R3, etc.

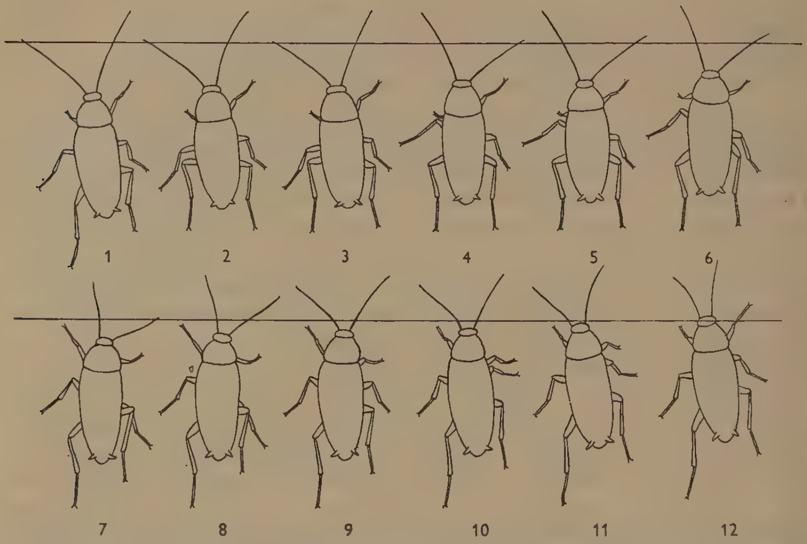
Such a rhythm is of rare occurrence especially beneath the photoflood lighting. It has been frequently observed in slowly-moving cockroaches, however, and in slowly moving insects such as aphids after they have been kept at low temperatures. A similar rhythm was also noted by Bert (1866) in dragonflies. In other parts of the film from which Text-fig. 6 was taken, the rhythm L3, L2, R3, L1, R2, R1, L3, etc. was also found.

From these observations it is concluded that the rhythm of leg movements during normal walking obeys the following two rules:

(i) No fore or middle leg is protracted until the leg behind has taken up its supporting position.

(ii) Each leg alternates with the contralateral limb of the same segment.

It will be noted that the first rule is exactly the same as that which Borelli (1680) suggested would be most advantageous for a hexapod. The rhythm which Paul Bert described for *Carabus auratus* also agrees with this rule, but he was mistaken in supposing that the diagonal middle and hindlegs move simultaneously.

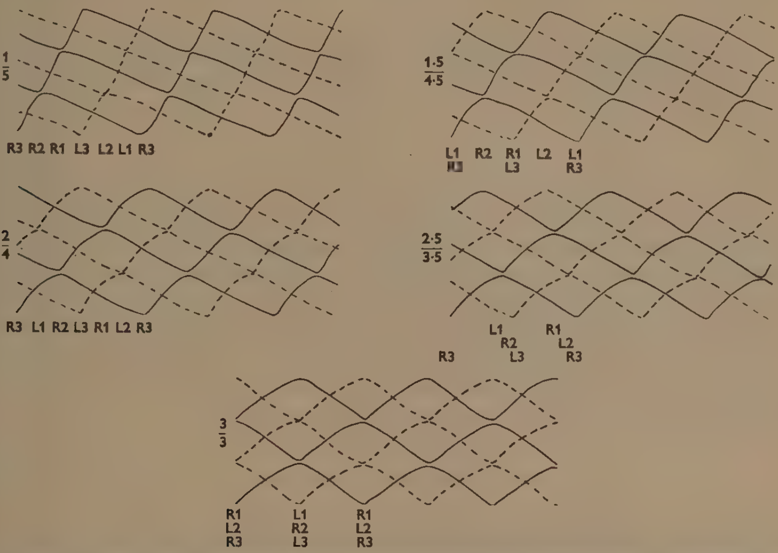


Text-fig. 6. Tracings of photographs from a film of *Periplaneta* walking at an exceptionally slow speed. The rhythm of protraction is R3, R2, R1, L3, L2, L1, etc. 16 frames/sec., every frame.

IV. *The changes in limb movements associated with an increase in speed*

The recognition of these two rules makes it easier to understand the relationship between the various rhythms discussed above. It can be shown (Text-fig. 7) that all of these rhythms may be obtained in a system obeying the two rules, and in which the ratio protraction time/retraction time (p/r) is varied. In Text-fig. 7, p/r is varied but $p+r$ remains constant. During insect walking $p+r$ tends to decrease with an increase in speed, but this does not alter the effect of the increase in p/r. Thus, in the rhythm L3, L2, L1, R3, R3, R1, which is only found when the insects move very slowly, only one leg is off the ground at any one instant and p/r is about 0·2. If the insect moves faster as a result of a decrease in retraction time which is relatively greater than the decrease in the time for protraction, p/r will increase and a rhythm similar to that of *Chrysomela* will be followed. It can be seen that with an increase in speed the ratio tends towards unity when a rigid alternation of tripods would result and this was the system stressed by earlier workers. It has been pointed out, however, that a slight delay remains between the protraction of the three legs of a tripod even when the insect is moving very fast, and corresponding to this p/r is never quite unity.

An alternative to this system is one in which both p and r are shortened, but p/r and hence the rhythm remains constant at all speeds. In tetrapods such as the newt and in the cockroach, however, an increase in speed is always accompanied by a relatively greater decrease in the duration of retraction than of protraction. The energetics of the muscles involved may determine this feature as such a system is probably the most efficient.



Text-fig. 7. Graphs to show the effect of variations in the ratio protraction time/retraction time (the duration of protraction + retraction remaining constant) on the rhythm of leg movements in a hexapod which follows the two rules: A, no fore or middle leg is protracted until the leg behind has taken up its supporting position; B, each leg alternates with the contralateral leg of the same segment.

The rhythm L3, L2, L1, etc., is rarely found, and then only at speeds below 1 cm./sec., but it is very important as it stresses the basic rules of the walking rhythm. Cockroaches use very nearly the same rhythm (R1, L2, R3, etc.) at all speeds from 1 to 25 cm./sec., as was found by recording the rhythm for individual cockroaches moving at over a dozen different speeds. Graphs showing the rhythm at two different speeds are shown in Text-fig. 5B and C. The increase in speed is accompanied by a quickening of both the protraction and retraction phases of the cycle, and p/r increases (see Table 1). There is also a tendency for a decrease to occur in the stride length, i.e. the movement of the limb tip relative to the head between complete protraction and maximum retraction. The distance between successive *points d'appui* was determined from the films, and by timing the insect walking over a measured distance of smoked paper and then measuring the distance between successive 'footprints'.

From these observations it appears that the same basic rhythm of leg movements is found at all speeds of cockroach movement, although at the very slowest ones different rhythms may be observed. But these grade insensibly into the normal rhythm with an increase in speed, and no evidence has been found for a distinction between running and walking among insects. It is true that a slowly-moving cockroach immediately moves at a quicker speed when it is suddenly startled. From this observation it might be concluded that these insects are essentially two-speed mechanisms, but this cannot be maintained because many intermediate speeds are also found. This conclusion is contrary to that of Pringle (1939), who suggested that it was physiologically justifiable to distinguish walking and running. This suggestion was based on the description of a double innervation of insect muscle, but recent histological and physiological work (Hughes, 1952) suggests that several cockroach leg muscles are innervated by at least three motor nerve fibres.

Table 1

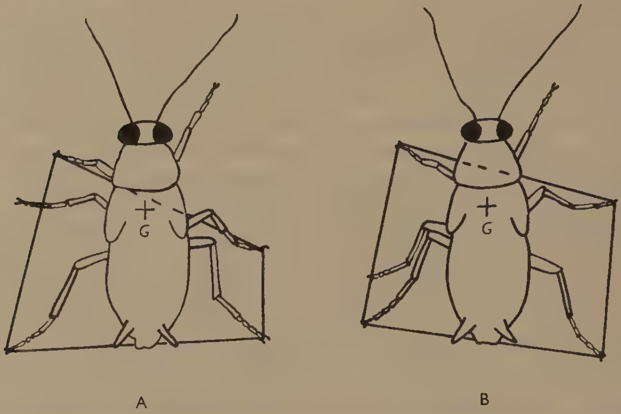
Speed (cm./sec.)	Protraction time / Retraction time	Stride length	Distance between *points d'appui*
17·5	0·8	10·0	23·0
9·3	0·5	11·6	22·0
5·0	0·35	13·4	19·6
3·2	0·31	13·5	19·0

CONCLUSIONS

The structure and movements of the legs of insects show considerable adaptive modifications so that a description based upon their condition in the cockroach cannot be extended to the whole class. It appears, however, that the rhythm of leg movements is remarkably constant for most orders of walking insects, and this feature strengthens the value of generalizations drawn largely from a study of cockroach movement. The constancy of the rhythm throughout insects, and the observation that the same rhythm is found in other Arthropods when these are rendered hexapodal suggests that it may possess advantages over the other possible sequences of leg movements. A constant pattern of walking movements is also found among Tetrapods, and here Gray (1944) has shown that the rhythm is determined by the mechanics of a four-legged system. He showed from theoretical considerations that it is the only one of the six possible rhythms in which stability is preserved throughout the cycle of leg movements. It is noteworthy that this same rhythm is found in insects which normally walk with four legs. For example, praying mantids use the rhythm L2, R3, R2, L3, L2, etc., when moving slowly with the front pair of prehensile legs off the ground. Similarly, some grasshoppers, and particularly the hopper stages of locusts, in walking adopt the rhythm L1, R2, R1, L2, L1, etc., while the hindlegs are lifted completely off the ground or move in a relatively independent rhythm, particularly when the insect is climbing (von Holst, 1943). The change in rhythm following the amputation of legs in the cockroach will be discussed in a subsequent paper, but it is relevant to point out here that the rhythm of a four-legged cockroach again conforms with that of walking Tetrapods.

A slowly-moving hexapod can move one leg at a time in many different sequences and retain stability throughout the cycle. It is evident that with an increase in speed either the rhythm may remain constant but be speeded up uniformly throughout the cycle, or the gait may change as a result of the unequal shortening of different parts of the cycle. Few workers have recognized different gaits in invertebrates, but Manton (1950) has recently given an account of many different gaits in *Peripatus*. She writes: 'Changes in speed of progression in *Peripatus* are carried out largely by an alteration in the gait, there being little change in the duration of each pace. In the *Myriapoda*, on the contrary, changes in speed are mainly effected by alterations in the pace duration, the gait remaining the same.' Insects conform to the myriapodan plan in so far as a shortening of the pace duration accompanies an increase in speed. It has been shown, however, that there are apparent changes in gait which merge into one another, and are the consequence of an increase in the ratio protraction time/retraction time at faster speeds, together with a close adherence to the two basic rules. In insects moving at faster speeds the legs move at almost equal intervals of time after one another. Such a rotary system produces a more uniform velocity than one of strictly alternating tripods, and it is probable that the muscles function more efficiently under the former conditions, particularly at the slower speeds. As in *Peripatus*, the gaits at faster speeds ensure fewer contacts with the ground at any one instant. The same is true of the horse in rapid motion, but the insect never has fewer than three legs on the ground, and hence even at the most rapid speeds it is mechanically stable should it stop at any instant during the cycle of movements. As this static stability cannot be achieved with fewer than three legs on the ground, it is apparent that in insects contacts with the ground have been reduced to the minimum number with which this is possible. This feature of their locomotory mechanism is no doubt responsible, in part, for the 'alertness' which we associate with all insects, as it enables them to stop suddenly or to change direction without needing to slow down.

This account has largely substantiated Borelli's view that the three ipsilateral legs should be lifted in the order hind, middle, fore. The only other possibility is one in which the wave of protraction moves backwards, and it can be shown that this has several disadvantages. If we assume that the order of protraction was fore, middle, hind, then it is evident that the rule would be that no leg should move before the leg in front had taken up its supporting position. This is obvious on considering the stage when the foreleg is off the ground, because otherwise the middle leg would also be lifted and the almost fully retracted hindleg would be quite insufficient to support the weight of the body on that side of the insect. The rhythm R3, L2, R1, L3, R2, L1, R3 obeys the rule, and is the reverse of the normal one. Such a rhythm would be stable at slow speeds, but it has the inherent disadvantage that the powerful propulsive stroke of the hindleg takes place before the more anterior parts of the supporting base have been established, and for this reason it might be unstable at more rapid speeds. This is most clear if we again consider the instant at which R1 is lifted. At this moment L2 will be about to be placed on the ground and R3 will be exerting its maximum lateral thrust. In the other triangle L1 will be half retracted, R2 more retracted and L3 almost fully retracted as it would be the next leg to protract. The area of support is depicted in Text-fig. 8B, where it can be seen that the anterior edge of this is determined by L1 and R2, both of which are more than half retracted. Furthermore, R3 is exerting its maximum thrust, and there is no support in front of the centre of gravity on this side of the body where the need is probably greatest because of the turning moment resulting from the activity of the hindleg. Contrast this with the condition in the normal rhythm (Text-fig. 8A) where fore and middle legs of a given triangle have both commenced their active strokes and are in a supporting position before the hindleg exerts its maximum thrust. This system greatly lessens the danger of the centre of gravity falling outside the area of support even at the most rapid speeds and its advantage in bringing about a more rectilinear motion has already been considered.



Text-fig. 8. Diagrams to show the area of support in a cockroach when the right foreleg is lifted off the ground. A, insect using the normal rhythm. The centre of gravity (*G*) is well within the area of support; B, insect in which the rhythm is reversed. The leg positions in this case were defined by reference to photographs of the limb pairs in the normal rhythm at the appropriate phase shown on graphs for the reversed rhythm.

From these considerations it appears that not only does the possession of six legs enable one pair to be adapted for another purpose, but also their rhythm is such that the animal can grade its speed, using presumably the same neuromuscular mechanism, and automatically reduce the number of contacts with the ground while retaining static stability at all speeds.

SUMMARY

1. An account is given of the functional morphology of the coxal articulations in the cockroach. The greater range of movements executed by the prothoracic legs is made possible by the mobility of the trochantinal condyle relative to the episternal condyle.

2. The movements of the individual legs were studied by means of films taken at speeds of 16–32 frames/sec. These show that more than three legs are in contact with the ground throughout the cycle of movements at normal speeds. Suggestions are also made concerning the mechanics of walking, but these require experimental verification.

3. The rhythm of walking movements in *Periplaneta*, *Blatta*, *Dytiscus*, *Hydrophilus*, *Carabus*, *Blaps* and *Chrysomela* obeys two rules: (i) no foreleg or middle leg is protracted until the leg behind has taken up its supporting position; (ii) each leg alternates with the contralateral one of the same segment. Other pterygote insects appeared not to differ from the species studied in any essential feature.

4. An increase in speed is accompanied by a decrease in the times of both protraction (p) and retraction (r), a shortening of the stride length, and an increase in the distance between the successive *points d'appui*. The range of speeds is continuous and no distinction could be recognized between walking and running.

5. Several gaits have been observed, the most common order of protraction being R1, L2, R3, L1, R2, L3, R1, etc., but these grade into one another if the ratio p/r is altered and the two rules obeyed. A system of rigidly alternating tripods would result if the ratio p/r was unity, but this is never quite realized as there is always a delay, sometimes extremely short, between the protraction of the three legs of a triangle. At very slow speeds the rhythm R3, R2, R1, L3, L2, L1, R3, etc., may be present.

6. It is concluded that insects are the end-product of a process of limb reduction among terrestrial Arthropoda in which p/r may be increased to nearly one and yet the animal retains static stability throughout the whole cycle. This is impossible with fewer than six legs.

This work was done during the tenure of a Martin Thackeray Studentship at King's College, Cambridge, and a Research Training Grant of the Department of Scientific and Industrial Research. I am greatly indebted to Prof. J. Gray, F.R.S., for suggesting the problem and for supervising this research. I also wish to express my thanks to Mr K. C. Williamson for his assistance with the cinematography.

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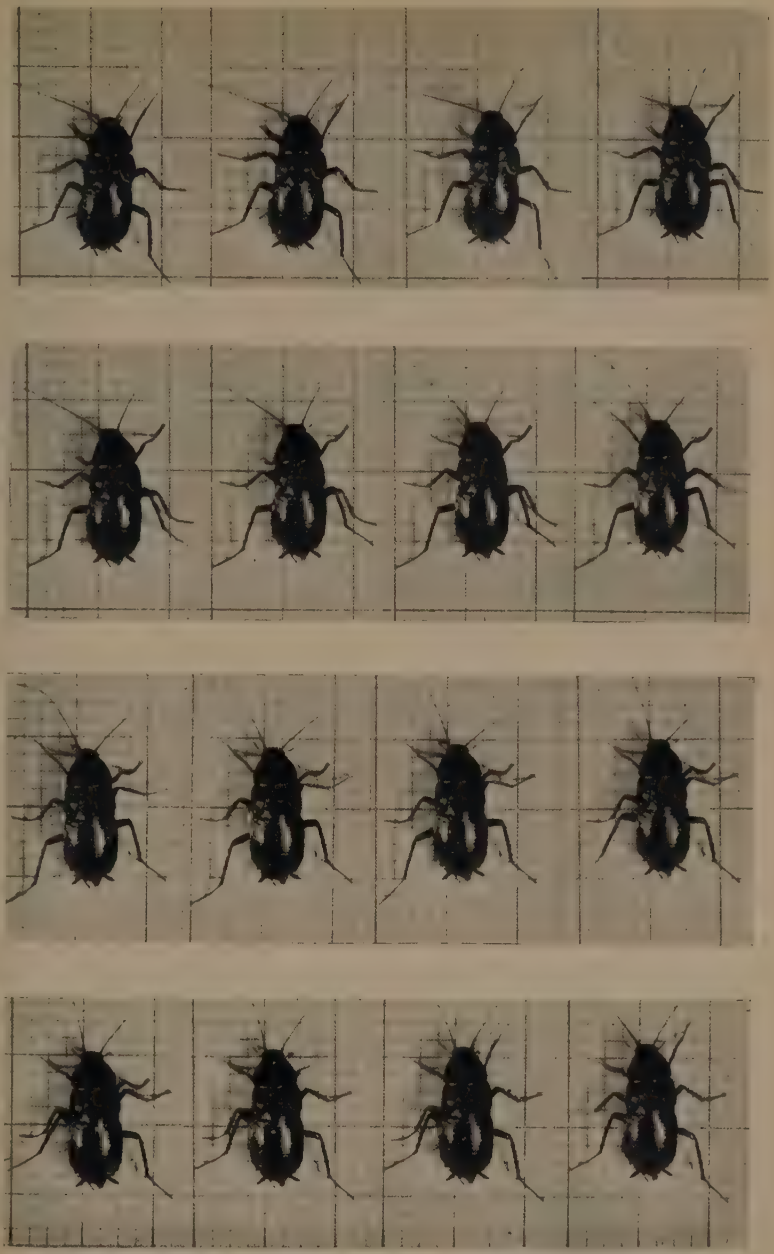
EXPLANATION OF PLATE 12

A series of photographs from a ciné film of *Blatta* during normal walking. The rhythm of protraction is R1, L2, R3, L1, R2, L3, R1, etc., 24 frames/sec., every frame.



HUGHES—THE CO-ORDINATION OF INSECT MOVEMENTS

THE METABOLISM OF THE SEA-URCHIN EGG

ANAEROBIC BREAKDOWN OF CARBOHYDRATE

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(*Received* 23 *October* 1951)

(With One Text-figure)

INTRODUCTION

Although a knowledge of resting egg metabolism is essential for an understanding of the changes in metabolism which occur at fertilization, opinions are still divided regarding the pathways of carbohydrate breakdown in the sea-urchin egg. A detailed examination of these differences of opinion has recently been published (Rothschild, 1951), so here it need only be said that the Swedish school, notably Lindberg & Ernster (1948), believe that in the sea-urchin eggs, carbohydrate is mainly broken down by an oxidative mechanism known as the hexose monophosphate shunt (Warburg & Christian, 1932; Dickens, 1936, 1938*a*, *b*) and not through the classical glycolytic pathways. The evidence in favour of this hypothesis cannot be considered conclusive, while Ycas (1950) has described experiments which support the opposite view, that sea-urchin egg carbohydrate is broken down by the conventional glycolytic mechanisms. The object of the work described in this paper was to try and resolve these differences of opinion, using the unfertilized eggs of *Echinus esculentus*. The eggs of *E. esculentus* will be shown to contain active glycolytic enzymes and to produce lactic acid under anaerobic conditions.

MATERIAL AND METHODS

Eggs were obtained from the ovaries by one of two methods: (*a*) Separated ovaries were cut up and the contents suspended in about 5 vol. of sea water by gentle agitation. The suspension was strained through bolting silk, and centrifuged; the supernatant was removed by suction and the eggs were resuspended in about 50 vol. of sea water. After the eggs had settled, the supernatant was removed by suction. (*b*) Normal and rapid extrusion of the eggs through the genital pores was induced by placing cotton-wool soaked in 0·5 M-KCl on the ovaries *in situ*. These eggs were centrifuged and washed once as described above.

Jelly was removed by centrifuging a concentrated egg suspension in an angle centrifuge at approx. 7000 × *g*, after which the jelly layer could be removed by suction. Alternatively, 0·15 N-HCl in sea water was added to a 20% suspension of eggs in sea water, the volume ratio of HCl in sea water to egg suspension being 1 : 10.

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After 5 min., during which the suspensions were occasionally agitated, the eggs were washed twice with 60 vol. of sea water.

More than 95% of the eggs prepared by these methods showed normal membrane elevation after fertilization. In a few cases fertilized eggs were observed up to the first cleavage stage. In these, 80–90% of the eggs underwent normal cleavage.

For the preparation of homogenates or egg extracts, eggs packed by centrifugation (angle centrifuge, 7000 × *g*) were suspended in an equal volume of M/50-phosphate buffer, pH 7·5, which caused complete cytolysis. Extracts were prepared by centrifugation of homogenates, the opalescent supernatant being used as the enzyme preparation.

Experiments were carried out in Thunberg tubes, the enzyme being placed in the side-arms. The tubes were evacuated to 7 cm. Hg and filled with N_2 which had previously been passed over hot Cu. After six cycles of evacuation and filling with N_2, the tubes were placed in a water-bath at 20° C. and, after temperature equilibration, the contents mixed by tipping. One ml. of enzyme preparation, the extract equivalent of 0·5 ml. eggs, was used in a final volume of 2–2·5 ml.

Lactic acid was estimated by the method of Barker & Summerson (1941), and pyruvic acid by that of Friedmann & Haugen (1943).

The substances added to the egg extracts (forming 'complemented extracts') were from the following sources: glycogen (rabbit liver), BDH; soluble starch, Hopkins & Williams' Analar; cozymase (DPN), purity 21% by the spectrophotometric method of LePage (1949), Light and Co.; adenylic acid, Light and Co.; adenosinetriphosphate (ATP), 60% pure on P_7, P total and N_2 analysis, the contaminant being inorganic, Light and Co.; hexose diphosphate, Ca salt of 85% purity by easily hydrolysable and total P analysis, Light and Co.

Because of the sensitivity of sea-urchin egg homogenates to Ca (Hultin, 1950), the hexose diphosphate was converted to the Ba salt by three cycles of solution in N/5-HCl and precipitation at pH 8 in the presence of a large excess of Ba and 0·15 vol. ethanol. The resulting product was 95% pure on easily hydrolysable and total P analysis.

Analytically pure glucose-1-phosphate was prepared by the method of Sumner & Somers (1944). Fructose-6-phosphate was prepared from hexose diphosphate by that of Neuberg, Lustig & Rothenberg (1943). It was 97% pure on P total and fructose analysis. We are indebted to Prof. F. Dickens, F.R.S., for gifts of glucose-6-phosphate and phosphogluconic acid (purity > 95%) and to Prof. E. Baldwin for a gift of phosphoglyceric acid (purity > 95%).

All phosphorylated intermediates were in the form of Ba salts. The appropriate weight of each was dissolved in N/5-HCl and the Ba precipitated with slightly more than the theoretical quantity of Na_2SO_4. The $BaSO_4$ precipitate was washed once with N/5-HCl, the combined supernatants brought to pH 7·5 and diluted to a final concentration of M/20. All other solutions were brought to pH 7·5 before use.

Glycogen was estimated in jelly-free eggs by the alkaline hydrolysis and precipitation methods of Lindberg (1945). After 30 min. hydrolysis in 5N-H_2SO_4 (see Sahyun, 1933) and neutralization to litmus, the reducing sugar was determined by the method of Folin & Malmros (1929). Total lipid was estimated by extracting packed eggs three times with 7% tricholoroacetic acid (TCA) and washing with 0·5% TCA. The residue was treated once with 8 vol. cold ethanol, once with boiling absolute ethanol under reflux and once with a 3 : 1 ethanol-ether mixture under reflux. The extracts were evaporated to dryness, extracted three times with boiling chloroform, filtered and the dry weight of the chloroform extract determined. Aliquots of the chloroform extract were used for estimation of total P to give the phospholipid content.

RESULTS

Egg substrates. A sample of jelly-free eggs contained 3·6 mg. glycogen per ml. packed eggs. The dry weight of 1 ml. of packed eggs being 245 mg., the glycogen content was 1·5% of the dry weight. A sample of 20 ml. packed eggs contained 1·02 g. lipid, corresponding to a lipid content of 20% of the dry weight. 24% of the total lipid was phospholipid, on the assumption that the P-content of phospholipid is 4%.

Lactic acid production by whole eggs. After an induction period of about 1 hr., whole eggs produce lactic acid under anaerobic conditions (Fig. 1). No appreciable amounts of pyruvic acid are produced in these circumstances, while there is no lactic acid accumulation in the presence of oxygen. The induction period for anaerobic lactic acid production is not caused by the presence of small amounts of oxygen as it occurs in egg suspensions which have been subjected to prolonged gassing with N_2 passed over heated Cu. When the eggs begin to produce lactic acid in large amounts, microscopically visible signs of injury, in the form of cytolysed eggs, occur; the fertilizability of eggs is lost before cytolysis. By the time lactic acid production begins to decline, there is marked evidence of cytolysis.

Lactic acid production is inhibited by fluoride, iodoacetate, and phenylmercuric nitrate (Table 1). The plasma membrane may not be equally permeable (or impermeable) to each of these inhibitors, a factor which probably has an important influence on the results obtained with different inhibitors (cf. Table 4).

Table 1. *Effect of inhibitors on acid production of whole eggs* (Echinus esculentus)

Each Thunberg tube contained 2 ml. of a 50% egg suspension. Incubation time, 4 hr.

Inhibitor ...	Nil	Iodoacetate	Phenylmercuric nitrate	Fluoride
Concentration...	—	10^{-2}M	10^{-3}M	10^{-2}M
Lactic acid (μg.)	300	41	248	121
Pyruvic acid (μg.)	3	0	7	0

Eggs in M/50-phosphate buffer produced more acid than those in M/50-phosphate buffer in 0·3 M-KCl, while the supernatants from homogenates were more active than whole homogenates.

Stimulation of glycolysis by cofactor addition. The sea-urchin egg glycolytic system is stimulated by the addition of cofactors (Table 2). Mg^{++} did not stimulate extract glycolysis.

Effect of different substrates on acid production. The experiment in Table 3 indicates that lactic and pyruvic acid production is increased by the addition of fructose and glucose, and to a lesser extent, by glycogen and starch.

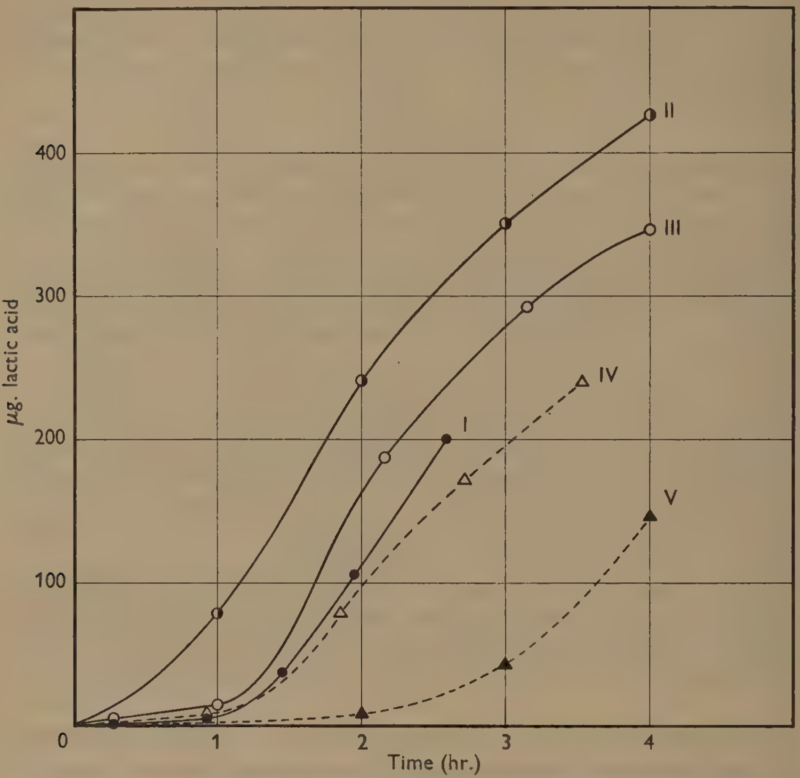

Fig. 1. Lactic acid production (μg./ml. eggs) in different samples of unfertilized eggs of *E. esculentus*. Curves I–IV refer to eggs kept at 20–21° C. during the experiment. The eggs in curve V were kept at 15° C. during the experiment and showed signs of injury during the third hour. The eggs in curve II were 3 hr. old before use and showed signs of injury after 1 hr.

Inhibitors. Inhibitors of glycolysis cause a marked inhibition of extract glycolysis (Table 4).

Effect of phosphorylated intermediates on glycolysis. The experiments in Table 5 show that the phosphorylated intermediates of the classical glycolytic system are also capable of yielding lactic and pyruvic acids in the sea-urchin egg system. The enzyme preparation used in the second experiment in Table 5 was made from eggs which had been left standing for 3 hr. before cytolysis. The experiment shows that under these conditions, the enzymes before aldolase, particularly phosphoglucomutase and

Table 2. *Effect of cofactor addition on acid production*

Each Thunberg tube contained M/20-phosphate buffer, pH 7·5 (final concentration). The additions were: glycogen, 12 mg.; cozymase (DPN), 200 μg.; ATP, 1 mg.; adenylic acid (AA), 1 mg. Exp. I, 1 ml. of 50 % extract, final volume, 2 ml. Exp. II, 1 ml. of 30 % extract, final volume, 2 ml. Incubation time, 60 min.

	System	Experiment I			Experiment II		
		Lactic acid (μg.)	Pyruvic acid (μg.)	Sum (μg.)	Lactic acid (μg.)	Pyruvic acid (μg.)	Sum (μg.)
(1)	Extract	−8	55	47	0	5	5
(2)	(1) +glycogen	28	119	147	3	8	11
(3)	(2) +DPN	244	14	258	44	27	71
(4)	(2) +ATP	66	147	213	7	6	13
(5)	(3) +ATP	252	34	286	59	67	126
(6)	(5) +AA	220	24	244	40	67	107
(7)	(5) without glycogen	193	17	210	40	45	85
(8)	(3) +AA	—	—	—	40	25	65

Table 3. *Comparison of acid production with different substrates*

Each Thunberg tube contained 1 ml. of 50 % extract, 200 μg. DPN, 1 mg. ATP, M/20-phosphate buffer, pH 7·5 (final concentration), final volume, 2 ml. Substrate concentration, M/40 (or M/40 glucose equivalent in the case of starch and glycogen). Incubation time, 60 min.

Substrate	Nil	Glycogen	Starch	Glucose	Fructose	Glucose-1-phosphate
Lactic acid (μg.)	137	235	273	334	337	275
Pyruvic acid (μg.)	93	149	147	162	176	234
Sum (μg.)	230	384	420	496	513	509

Table 4. *Effect of inhibitors on reconstructed glycolytic system*

Each Thunberg tube contained 1 ml. of 40 % egg extract, M/20-phosphate buffer, pH 7·5 (final concentration), 200 μg. DPN, 1 mg. ATP, and 12 mg. soluble starch, final volume, 2 ml. Incubation time, 60 min.

Inhibitor	Nil	Iodoacetate		Fluoride		Phloridzin	Phenylmercuric nitrate
Concentration...	—	10^{-2} M	10^{-3} M	10^{-2} M	10^{-3} M	10^{-2} M	10^{-3} M
Lactic acid, μg.	111	18	80	20	82	48	3
Pyruvic acid, μg.	78	4	5	−4	−36	62	8
Sum, μg.	189	22	85	16	46	110	11

hexose isomerase, enzymes shown to be present in sea-urchin egg extracts by Ycas (1950), are the most labile.

DISCUSSION

The glycogen content of the eggs of *E. esculentus* is lower than that of other species (Orström & Lindberg, 1940; Hutchens, Keltch, Krahl & Clowes, 1942), though it is about the same as that of oyster eggs (Cleland, 1950*b*). Oyster eggs, however, have a much lower glycolytic rate than those of *E. esculentus* (Table 6). The total lipid content of the latter is of the same order as in other species (Hayes, 1938; Ohman, 1944), although the phospholipid concentration is lower. The lack of visible lipid inclusions in the egg of *E. esculentus* is interesting in view of the high concentration of these substances.

The experiments described in this paper show that whole eggs are capable of anaerobic lactic acid production, which confirms the findings of Perlzweig & Barron (1928) and Ycas (1950). The time course of this lactic acid production is complex, the curve being more or less sigmoid, with an induction period of about an hour at 20° C. (cf. Dickens & Greville, 1932, 1933), and a declining rate of production after 2–3 hr. of anaerobiosis. Cytolysis at this stage might account for the decline in lactate production.

Having excluded the possibility that residual oxygen can account for the induction period, the following explanations of this phenomenon are worth consideration:

(1) There exists a labile or easily saturated mechanism which can degrade pyruvic or lactic acid. Such a mechanism has been described in oyster eggs by Cleland (1950*b*). In this material a system is present on the cytoplasmic granules which is capable of removing added pyruvate under anaerobic conditions. The effect is stimulated by CO_2 or bicarbonate.

(2) An intermediate product of glycolysis, which is not present, or present in limiting amounts, under aerobic conditions, is slowly produced. If this suggestion is correct, aerobic and anaerobic metabolism may proceed through different pathways.

(3) The egg injury which ultimately occurs under the conditions of the experiments may increase the rate of glycolysis.

Explanation (1) is unlikely to be correct because homogenates cannot degrade added pyruvate anaerobically. Explanation (2) is also unlikely to be correct since the possible limiting factors (inorganic P, ATP, and adenylic acid) have been found in other sea-urchin eggs. The third explanation is most likely to be the correct one. Damaged eggs often exhibit an increase in O_2 uptake (Rothschild, 1949; Runnström, 1949; Cleland, 1950*a*), while completely cytolysed eggs may have a higher rate of O_2 uptake than uninjured unfertilized eggs (Warburg, 1914). In these experiments, injury, as evidenced by cytolysis or loss of fertilizability, coincided with the onset of rapid lactic acid production and it is reasonable to expect a causal relationship between the two phenomena. This does not mean that no anaerobic lactate production occurs until the eggs are irreversibly damaged, but that injury causes an increased production. This distinction is quantitative rather than qualitative and, in the case of *Psammechinus miliaris*, the eggs are fertilizable after the maximum rate of anaerobic lactic acid production has been reached.

Acid production by egg extracts is stimulated by the usual cofactors of glycolysis, as is shown in Table 2. The slight inhibitory action of adenylic acid when added with ATP, and the lack of stimulation when it is used alone, may be due to it being present in adequate quantities in uncomplemented extracts, or to the presence of heavy metal impurities in the sample of adenylic acid used. The failure of added Mg to stimulate glycolysis does not imply that Mg is not a normal requirement of the system and that therefore the system is non-glycolytic, for the following reasons: first, because free Mg^{++} was present both in the egg homogenates, the concentra-

Table 5. *Acid production using phosphorylated intermediates as glycolytic substrates*

Each Thunberg tube contained 1 ml. 45 % extract, 200 μg. DPN, 1 mg. ATP, 1 mg. AA, M/20-phosphate buffer pH 7·5 (final concentration), final volume, 2 ml. Final substrate concentration, M/200. Incubation time, 45 min. in Exp. I, 60 min. in Exps. II and III.

Substrate	Experiment I				Experiment II				Experiment III			
	Lactic acid (μg.)	Pyruvic acid (μg.)	Sum (μg.)	Excess over control	Lactic acid (μg.)	Pyruvic acid (μg.)	Sum (μg.)	Excess over control	Lactic acid (μg.)	Pyruvic acid (μg.)	Sum (μg.)	Excess over control
Control	57	60	117	—	21	5	26	—	60	35	95	—
Glucose	121	90	211	94	28	8	36	10	143	55	198	103
Glucose-1-phosphate	101	139	240	123	33	7	40	14	122	68	190	95
Glucose-6-phosphate	95	157	252	135	55	26	81	55	136	108	244	149
Phosphogluconate	63	68	131	14	4	18	22	−4	61	39	100	5
Fructose-6-phosphate	103	252	355	238	56	117	273	247	176	174	350	255
Hexosediphosphate	136	248	384	267	136	196	332	306	205	232	437	342
Phosphoglycerate	61	670	731	614	29	750	779	753	57	755	812	717

Table 6. *Comparison of glycolysis in different invertebrate tissues*

Animal	Organ	Temp. (° C.)	System	Complement	*Q**	Reference
Cockroach	Muscle	25	Teased fibres	Cofactors and substrate	0·4	Barron & Tahmisian, 1948
	Muscle	37	Homogenate	Cofactors and substrate	0·67	Humphrey, 1949
Grasshopper	Muscle	30	Homogenate	Cofactors and substrate	2·7	Humphrey & Siggins, 1949
Oyster	Muscle	37	Extract	Cofactors and substrate	2·0	Humphrey, 1944
	Egg	25	Extract	Cofactors and substrate	0·5	Cleland, 1950*b*
Schistosoma mansoni	Whole worm	37	Entire	None	2·5	Bueding, 1950
				Substrate	35·0	Bueding, 1950
Sea-urchin	Egg	20	Whole egg	None	0·15	This paper
	Egg	20	Extract	Cofactors and substrate	1·7	This paper
Rat	Liver	37	Extract	Cofactors and substrate	20·0	Reiner, 1947
	Brain	37	Extract	Cofactors and substrate	60·0	Reiner, 1947
Chick	Embryo	37	Extract	Cofactors and substrate	7·0	Novikoff *et al.* 1948

* μl. lactic acid or lactic + pyruvic acid/hr./mg. dry weight. The figures are calculated from the most active preparations quoted.

tions of which were high, and in the small quantities of interstitial sea water; and secondly, because fluoride inhibited acid production.

The experiments in Table 2 show that in contrast to whole eggs, in which only lactic acid accumulates, complemented egg extracts produce both lactic and pyruvic acids. Furthermore, the lactate-pyruvate equilibrium varies in different experiments and the proportion of the two acids can be greatly affected by DPN addition. The accumulation of pyruvate as well as lactate is characteristic of glycolysis in extracts of invertebrate material (Humphrey, 1949); in oyster-egg extracts, pyruvic acid is virtually the only acid which accumulates (Cleland, 1950*b*). It has not yet been shown, however, that lactic and pyruvic acid accumulate in intact invertebrate cells.

The production by the extracts of lactic and pyruvic acid from polysaccharides and monosaccharides suggests that phosphorylase and hexokinase are present; but a high amylase activity might account for these results, in which case the presence of phosphorylase would be in doubt. The inhibition by phloridzin and the fact that glucose-1-phosphate is an efficient substrate favour the view that phosphorylase is present.

The experiments with inhibitors of glycolysis also suggest that a typical glycolytic cycle operates in the sea-urchin egg. Iodoacetate and fluoride, in the usual concentrations, depress the acid production of whole eggs and complemented extracts; while phenylmercuric nitrate, a powerful —SH inhibitor, has a marked inhibitory action. Phloridzin, which inhibits the acid production that occurs during the cytolysis of these eggs (Rothschild, 1939), also inhibits lactic acid production in complemented extracts.* Lindberg & Ernster (1948) attach some importance to their failure to inhibit the oxidation of glucose by iodoacetate, and regard this as confirmation of their view that glycolysis is not the principal mechanism for carbohydrate breakdown in the sea-urchin egg.† The responses to inhibitors of invertebrate enzyme systems are known to differ from those of mammalian systems. For example, iodoacetate does not inhibit lactic acid production in some insects (Barron & Tahmisian, 1948; Humphrey & Siggins, 1949), although a typical effect occurs in oyster eggs (Cleland, 1950). In any case, the experiments recorded in this paper show that iodoacetate does inhibit sea-urchin egg glycolysis, even though it may not inhibit the oxidation of glucose.

Apart from the evidence already given, the fact that the phosphorylated intermediates of mammalian glycolysis undergo rapid breakdown to lactate and pyruvate in sea-urchin egg extracts constitutes further and cogent evidence for the operation of a normal glycolytic cycle in this system. The three experiments cited in Table 5 show that the glycolytic rates with different substrates may vary from preparation to preparation, which might be expected in a complex system some of whose component enzymes are known to be labile. The small activity of phosphogluconic acid is probably due to slight contamination with glucose-6-phosphate. Sea-urchin eggs, like all eggs so far studied, have a low phosphatase activity (Connors & Scheer, 1947; Krugelis, 1947; Cleland, 1950*b*). It is therefore unlikely that dephosphorylation of the phosphorylated intermediates played any part in their observed effect on glycolysis.

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* It would be wrong to assume that the acid of injury, observed manometrically by Rothschild (1939), is lactic acid; it almost certainly is not.

† As these experiments were done in the presence of M/10 NaF, it is most unlikely that any glycolysis would have been observed.

These considerations lead to the conclusion that the sea-urchin egg possesses a glycolytic system whose activity is comparable with that found in other invertebrate tissues. Table 6 enables such a comparison to be made, the values for the sea-urchin egg being similar to those reported for a number of invertebrate muscles.

SUMMARY

1. The eggs of *Echinus esculentus* contain about 1·5% glycogen and 20% lipid (dry weight).

2. Whole eggs produce lactic acid under anaerobic conditions, the maximum rate of production being 0·15 μl. lactate/hr./mg. eggs (dry weight) at 20° C.

3. Lactic acid production is inhibited by fluoride, iodoacetate and phenylmercuric nitrate.

4. The supernatant from centrifuged egg homogenates prepared in M/50-phosphate buffer, pH 7·5, ('extract'), was found to be a satisfactory glycolytic enzyme preparation.

5. The production of lactic and pyruvic acid by such extracts is greatly stimulated by the addition of glycogen, cozymase and adenosine triphosphate, ('complemented extracts').

6. Complemented extracts glycolyse glucose and fructose with greater facility than glycogen or starch. With glucose or fructose as substrate, the rate of formation of lactate and pyruvate may reach 1·7 μl./hr./mg. eggs (dry weight) at 20° C.

7. Complemented extract glycolysis is inhibited by fluoride, iodoacetate, phenylmercuric nitrate and phloridzin.

8. Active extract glycolysis occurs with the following glycolytic intermediates as substrates: glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, hexose diphosphate and phosphoglyceric acid.

9. It is concluded that the sea-urchin egg (*E. esculentus*) possesses a typical glycolytic system, whose activity is comparable with that found in other invertebrate tissues.

One of us (C.) is greatly indebted to the Director and staff of the Chester Beatty Research Institute for their hospitality.

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STUDIES IN DIURNAL RHYTHMS

II. CHANGES IN THE PHYSIOLOGICAL RESPONSES OF THE WOOD-LOUSE *ONISCUS ASELLUS* TO ENVIRONMENTAL STIMULI

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(With Three Text-figures)

INTRODUCTION

In an earlier paper in this series, it was pointed out that diurnal rhythms are basically of two kinds: exogenous, a direct response to environmental changes, and endogenous rhythms which persist under constant conditions. For example, the West African millipede *Ophistreptus* sp. exhibits a 24 hr. periodicity under constant conditions for 19 days (Cloudsley-Thompson, 1951 *a*). Many species of animals show a combination of both types and such rhythms are termed 'composite' by Park (1949).

Endogenous rhythms are frequently correlated with environmental changes such as light, temperature and relative humidity. As they are not a direct response to these changes, however, Dr D. L. Gunn has suggested that it is preferable to refer to them as 'clues' rather than 'stimuli'.

Another factor to be considered is that although many species are active during a certain period of the day or night and are quiescent for the remainder of the 24 hours, some exhibit different kinds of activity at different times. Thus the water-skater *Gerris* spends the daytime on the surface of ponds and streams, but flies from one locality to another at night (Riley, 1925); many other aquatic insects such as beetles, fly mostly at night, but swim actively throughout the day.

Most of the work on rhythmic behaviour in animals has, in the past, been concerned with establishing the existence of 24 hr. periodicities, and determining the 'clues' with which they are correlated (Calhoun, 1944). In the case of locomotory rhythms, little attention has been directed to the changes in physiological response which may be correlated with outbursts of activity. An example of this kind of change is afforded by the pill-woodlouse *Armadillidium vulgare*. This species is more resistant to desiccation than other species of Isopoda (Edney, 1951), and shows locomotory activity principally in the morning when it is often to be seen walking about in the sunlight (Cloudsley-Thompson, 1951 *c*). This is probably correlated with the fact that in this species some individuals exhibit positive phototaxis when the temperature rises (Henke, 1931).

The majority of woodlice such as *Oniscus* and *Porcellio* spp. are nocturnal, however, and come out at night from their hiding places under stones, logs and bark, and wander in dry places where they are not found during the day. For example, they are often to be seen climbing up walls after dark. Atmospheric humidity is greater during the night, the temperature drops and light is absent. In this paper an attempt has been made to indicate how nocturnal changes in environmental conditions may alter the daytime reactions of *Oniscus asellus*, and thus engender the observed locomotory activity.

MATERIAL

The species used in this investigation was *Oniscus asellus* L. collected from my garden in Esher, Surrey. Adult animals of both sexes were used, but these were not separated.

ESTABLISHING THE RHYTHM

Diurnal periodic locomotory activity was investigated using the Aktograph apparatus described by Gunn & Kennedy (1936). Instead of the original long writing lever, however, a short rod was connected with a gymbal lever, and a clockwork barograph drum was employed as a kymograph in place of a 12 inch motor-driven drum.

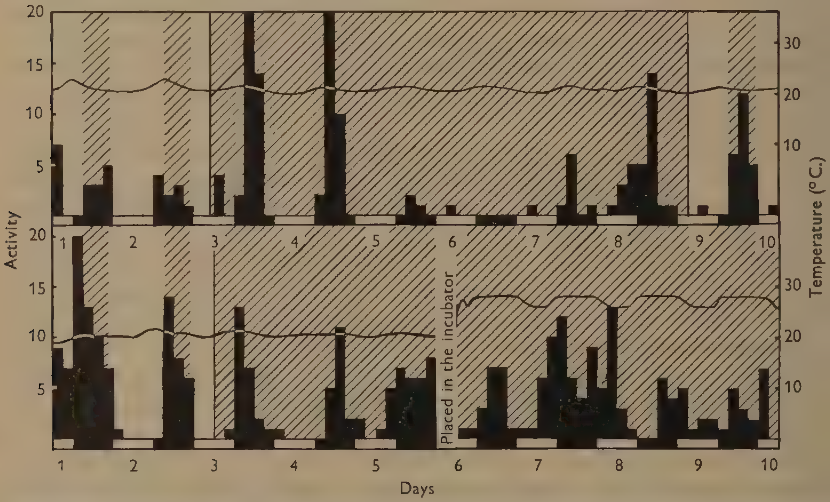

Fig. 1. Activity of *Oniscus*, in damp surroundings under natural lighting and in darkness at room temperature; and later in darkness at room temperature followed by artificially fluctuating temperature reaching a minimum during the day.

The object of these modifications was to reduce the overall size of the apparatus so that it could be placed in a large incubator, and light and temperature controlled artificially.

The floor of the arena was lined with damp filter paper so that the humidity was maintained at a high level, and did not fluctuate with temperature.

The results obtained are plotted as block histograms (Fig. 1), and show that locomotory activity at room temperature is mostly confined to the hours of darkness.

In preliminary experiments the arena was exposed to normal daylight and darkness, and fluctuating temperatures, the latter being registered on a recording thermometer. When, however, light was excluded, the periodicity disappeared after a few days, suggesting that the rhythm is 'composite', the 'clues' fluctuation in light rather than in temperature.

Fluctuations in temperature alone did not maintain the rhythm, but it was immediately resumed in alternating light and darkness.

Throughout the work it has been assumed therefore, that if woodlice are kept for several days in darkness, their normal periodicity is eliminated and the behaviour of such animals resembles that of control animals at night: check tests have confirmed this.

DIURNAL FLUCTUATIONS IN THE HUMIDITY RESPONSE

Experiments were carried out on *Oniscus asellus* by a modification of the method described by Gunn (1937), using two choice-chambers identical with those employed in the investigation of the humidity responses of millipedes (Cloudsley-Thompson, 1951*b*). One choice-chamber was kept in light, the other was darkened with a wooden cover. The air in one half of each arena was kept dry, using a mixture of sulphuric acid and distilled water calculated to give a relative humidity of 50 % (Buxton & Mellanby, 1934). On the other side, distilled water was placed beneath the perforated zinc gauze. A paper hygrometer recorded 60 and 90 % relative humidity respectively on the two sides of the arena. All experiments were carried out at room temperature 18 ± 2° C. in subdued daylight during March and April 1951.

The apparatus was set up overnight, and on the following morning five woodlice were placed in each arena. Their positions were noted at intervals of 15 min. Animals moving were counted separately, as were any within 1·5 cm. of the boundary. After each reading, the animals were stirred up with a glass rod so that after each stirring there were either two animals on each side and one in the middle, or three on the side which had previously had two.

After ten readings giving fifty position records, the animals were returned to the cultures. The apparatus was rotated between experiments so that external factors were cancelled out, and the positions of the perforated zinc platforms, and of the sides and lids were interchanged. The intensity of the reaction was so marked that on one occasion a slight leak of acid into the distilled water was immediately detected through the abnormal behaviour of the woodlice in the arena.

Two series of experiments were carried out. In the first, the responses in light of animals from a control culture exposed to daylight were compared with those of similar animals in the darkened choice-chamber. In the second series, the responses in light of control animals were compared with those of animals in the darkened choice-chamber which had previously been kept in darkness at room temperature for several weeks (Table 1).

The intensity of the reaction was calculated in each case by dividing the number of stationary woodlice on the moist side of the arena plus half the number in the middle, by the number on the dry side plus half the number in the middle. It was also calculated using Gunn's (1937) method in which those in the middle are omitted; but since they were considerably more numerous in the experiments carried out in darkness, they cannot in this case justifiably be ignored.

Table 1. *Number of woodlice moving or stationary on the dry* (50 % R.H.), *middle or moist* (100 % R.H.) *sides of humidity choice-chamber apparatus.*

	Totals of first halves of experiments	Percentages of first halves of experiments	Totals of second halves of experiments	Percentages of second halves of experiments	Grand totals	Percenta of gran totals
A. Moving	113	22·6	87	17·4	200	20·0
On dry side	21	4·2	13	2·6	34	3·4
In middle	49	9·8	24	4·8	73	7·3
On moist side	317	63·4	376	75·2	693	69·3
Total	500	100	500	100	1000	100
Intensity of reaction (Gunn's method)	—	15·1	—	28·9	—	20·4
Intensity of reaction (alternative method)	—	7·5	—	15·5	—	10·4
B. Moving	23	9·2	17	6·8	40	8·0
On dry side	32	12·8	3	1·2	35	7·0
In middle	31	12·4	16	6·4	47	9·4
On moist side	164	65·6	214	85·6	378	75·6
Total	250	100	250	100	500	100
Intensity of reaction (Gunn's method)	—	5·1	—	71·3	—	10·8
Intensity of reaction (alternative method)	—	3·8	—	20·2	—	6·9
C. Moving	7	2·8	9	3·6	17	3·4
On dry side	46	18·3	7	2·8	53	10·6
In middle	54	21·6	33	13·2	86	17·2
On moist side	143	57·3	201	80·4	344	68·8
Total	250	100	250	100	500	100
Intensity of reaction (Gunn's method)	—	3·1	—	28·7	—	6·5
Intensity of reaction (alternative method)	—	2·3	—	9·3	—	4·2

A, controls in light; B, controls in darkness; C, animals in darkness from a culture kept in darkness for se days previously. Room temperatures 18 ± 2° C.

From the results (Table 1) it can be seen that not only was the intensity of the humidity reaction of woodlice in darkness (B) considerably less than in controls (A), but it was still further reduced in animals (C) which had been kept in darkness for some days prior to the experiments. The larger number moving about in the choice-chamber in daylight (A) is due to their kinetic and tactic responses to light (Abbott, 1918; Dietrich, 1931; Henke, 1930), and the figures in the second and fourth columns show that the intensity of the reaction increased as the animals became progressively desiccated. In addition, this increase was considerably greater in controls in darkness (B) than it was in the animals from the dark culture in darkness (C).

DIURNAL FLUCTUATIONS IN THE RESPONSE TO LIGHT

In a study of the reactions to light of *Oniscus asellus* and two species of *Porcellio*, Abbott (1918) showed that the animals react negatively by means of both photokinesis and phototaxis, and that the response is the same at all intensities. In consequence in the experiments described below, no attempt was made to standardize the light intensity, diffuse daylight or darkness being offered on either side of the choice-chambers. In addition, Abbott suggested that *Porcellio* became somewhat less negative after living in a dry habitat, but that the reaction of *Oniscus* was essentially the same whether the animals had previously been exposed to strong light or to dark, and whether kept in a maximum or minimum of moisture. Waloff (1941), however, found a reversal from negative to positive phototaxis in *Oniscus* correlated with water loss by evaporation, and this was confirmed in the present work. Fraenkel & Gunn (1940) have shown that there is no need to postulate skototaxis in interpreting the results of Dietrich (1931) and Henke (1930) on the reactions of woodlice to light, since their orientation can be explained adequately in terms of negative phototaxis.

The responses of *Oniscus* from control cultures exposed to daylight were compared, as before, with those of animals from a culture kept in darkness. One half of each choice-chamber was darkened with a cover, the other was exposed to diffuse daylight. Ten woodlice were placed in each arena, and the number on the light side was noted at intervals of 15 min. The animals were stirred up after each reading, the same precautions being taken as before, and the dark cover was moved to the other half of the chamber.

In one set of experiments the floor of the arena was of damp filter-paper, in another the floor was of voile on zinc gauze covering a mixture of sulphuric acid and distilled water calculated to produce a relative humidity of 50 % (Buxton & Mellanby, 1934). The experiments were carried out at room temperature, 19·5 ± 2° C., during June and July 1951. At higher temperatures the differences in the responses seemed to be less clear. The results obtained are given in Fig. 2 from which it can be seen that animals from the dark cultures were more strongly photo-negative than the controls, and they tended to remain photo-negative at 50 % relative humidity whereas the controls became more positive in dry air.

DIURNAL FLUCTUATIONS IN THE RESPONSE TO CARBON DIOXIDE

The response to carbon dioxide of woodlice from control cultures exposed to daylight was compared with that of animals kept in darkness for a week or more. Groups of ten woodlice were placed in a crystallizing dish lined with damp filter-paper and covered with a lid of Perspex having an inlet and outlet for air. The air was pumped first through a concentrated solution of potassium hydroxide to remove all traces of carbon dioxide, then bubbled through water and passed into the crystallizing dish from 15 to 30 min. until all the animals had come to rest. Carbon dioxide from a cylinder was then bubbled through water, and allowed to enter the air stream in controlled amounts. The approximate concentration entering the crystallizing dish was ascertained by comparing the number of bubbles of carbon dioxide per minute with the number of air bubbles (300). The maximum number of woodlice stimulated into activity (exhibited either by locomotion, or by intense movements of the antennae) in a period of 5 min. was plotted graphically (Fig. 3).

Two sets of experiments, one with controls, the other with animals from cultures kept in darkness, were carried out concurrently in February and March 1951, at room temperature, 16 ± 1° C., between 10·00 and 16·00 hr. daily. The arena was

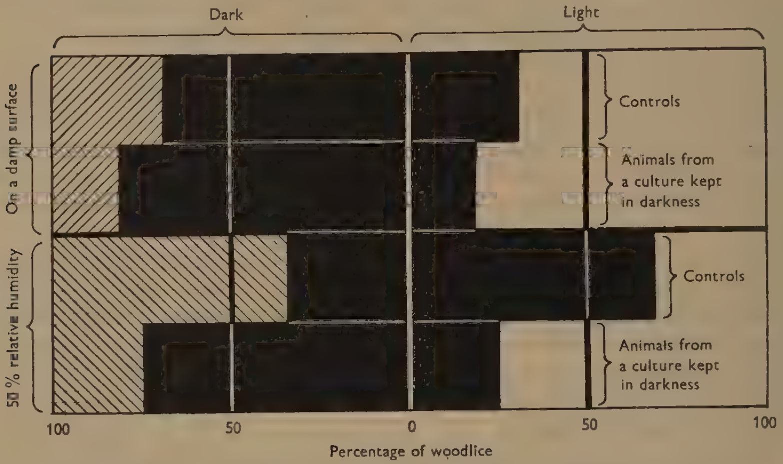

Fig. 2. Response of *Oniscus* to light, on a damp surface and at 50 % relative humidity. Controls and animals from a culture kept in darkness for some days previously.

exposed to daylight, but was considerably shaded, for animals from the dark cultures became very active in brighter light and would not otherwise come to rest. The woodlice were changed after one or two readings, because it was found that after an initial outburst of activity they frequently failed to respond to continued carbon dioxide. The response persisted after removal of the antennae.

From the difference between the two regression lines in Fig. 3, it is obvious that animals which had been kept in darkness were more sensitive to carbon dioxide than were the control animals; and check tests after dark showed that the latter were much more sensitive at night than during the day.

DISCUSSION

These experimental results are readily interpreted in relation to the ecology of the species. Woodlice spend the daytime under stones, logs, and in other damp, dark places. If an animal shows such a tendency to aggregate in particular localities as a result of physiological responses, it is reasonable to expect a reversal or modification of these responses at some time or other to account for the dispersal of the species. The experiments described above give an indication of the changes tha occur at night in the physiological responses of *Oniscus asellus* and are responsible for the nocturnal behaviour of this species.

A composite diurnal rhythm is correlated primarily with alternating light and darkness, and not with fluctuating temperature or humidity. Although none of these environmental factors is likely to vary much in the sheltered places where the animals aggregate during the day, the endogenous component of the rhythm will engender locomotory activity in some individuals at least, at nightfall. Even if the majority are exposed to daylight only occasionally, this 'clue' may be sufficient to keep their periodicity in phase with the 24 hr. cycle.

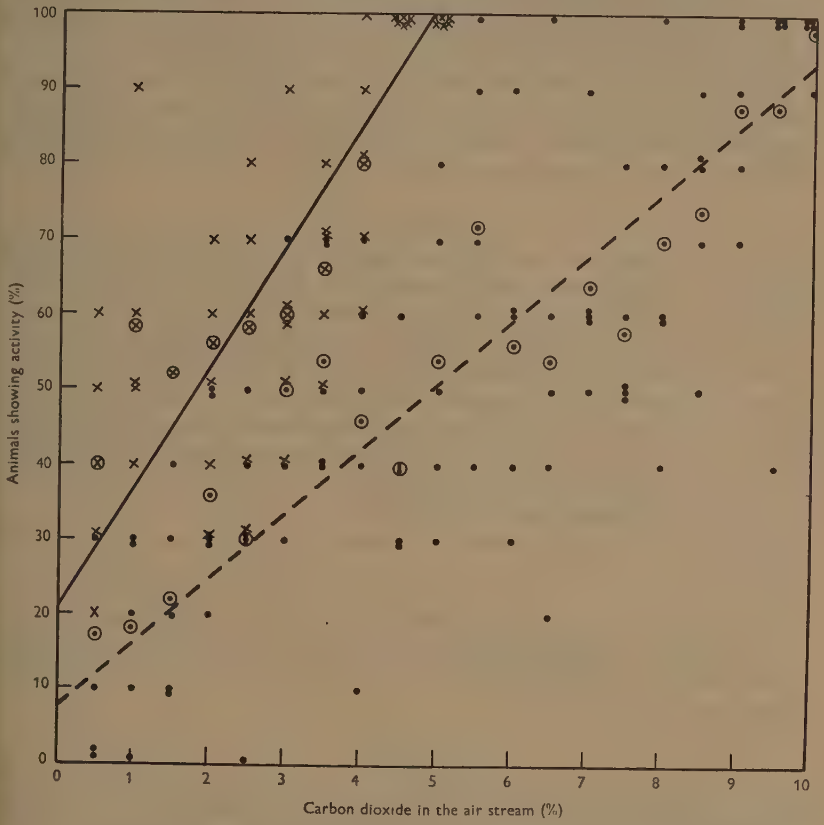

Fig. 3. Response of *Oniscus* to carbon dioxide. Controls ●– – – –● (means ⊙). Animals from a culture kept in darkness for some days previously ×————× (means ⊗).

Although the carbon dioxide in the atmosphere does not fluctuate greatly from day to night, the increased sensitivity of the responses to this gas under experimental conditions provides an illustration of the fact that woodlice are more responsive generally at night, are more easily disturbed then, and more readily exhibit locomotory activity.

The intensity of their humidity responses decreases at night, at any rate until the animals become somewhat desiccated, and this permits them to walk in drier places than those in which they pass the day; and the increased photo-negative response in darkness ensures that they get under cover at daybreak. In this way no doubt many potential predators are avoided. Perhaps this increase in the light response at night could be regarded as a kind of conditioning to darkness.

On the other hand, if their daytime habitat should dry up, the woodlice are not restrained there until they die from desiccation, for they become photo-positive in dry air and thus are able to wander in the open until they find some other damp hiding place when they again become photo-negative.

SUMMARY

A composite diurnal locomotory rhythm has been established in the woodlouse *Oniscus asellus*. This is correlated primarily with alternating light and darkness, and not with fluctuating temperature or humidity.

The intensity of the humidity response of the species is less in darkness than in light, and less still in darkness when the animals have been kept in darkness for some days previously. It increases with desiccation. The response to light is greater, too, in animals which have been kept in darkness for some days, and these tend to remain photo-negative in dry air whereas controls become photo-positive with desiccation. The sensitivity to carbon dioxide is much greater in animals from a culture kept in darkness than it is in controls.

The results obtained are discussed in relation to the nocturnal ecology of the species.

My thanks are due to Dr D. L. Gunn for the loan of his aktograph apparatus and for a number of stimulating discussions, to Dr G. P. Wells for suggesting the use of a barograph clockwork motor as a miniature kymograph, and to my wife for her constant help and advice.

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STUDIES ON TAPEWORM PHYSIOLOGY

VI. EFFECT OF TEMPERATURE ON THE MATURATION *IN VITRO* OF *SCHISTOCEPHALUS SOLIDUS*

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(With Fifteen Text-figures)

INTRODUCTION

It has been shown that the pleroceroid larvae of the bird cestode *Schistocephalus solidus* matures rapidly when cultured aseptically in suitable media at 40° C. provided pH and oxygen tension are controlled (Smyth, 1946, 1950). This temperature of 40° C. represents the average body temperature of birds, in the gut of which pleroceroids normally develop (Hopkins & Smyth, 1952).

It is the object of this paper to investigate the maturation of the pleroceroid at subnormal temperatures (i.e. $<40°$ C.) *in vitro*.

METHODS

Sticklebacks infected with pleroceroids were obtained from Roundwood Reservoir, and aseptic cultures prepared by the technique previously described. Horse serum + 1 % glucose was used throughout as a culture medium, 50 c.c. in 3 × 20 cm. plugged, rimless tubes. Scarcity of infected fish did not allow more than the following range of temperatures to be investigated: 40, 35, 34, 33 and 30° C. Ten larvae were cultured at each temperature.

At intervals during cultivation, larvae were removed and fixed for histological and cytological examination. Thus, a complete histological picture of the progress of maturation of the genitalia at different temperatures was obtained. Bouin, Carnoy, Flemming-with-acetic and 5% formol were used as fixatives; Carnoy being especially satisfactory for nuclear changes in spermatogenesis. In addition to the usual routine staining such as Heidenhain's Iron Alum Haematoxylin and Delafield's Haematoxylin and Eosin, Unna's Methyl Green Pyronin and Feulgen were particularly useful for demonstrating nuclear abnormalities that appeared at lower temperatures. Shell formation was followed by means of the specific method recently developed, using *aqueous* methyl green after formalin fixation (Smyth, 1951). In addition to sections aceto-carmine squashs and smears were used with some success.

Cultures were examined each day for egg production, and any eggs present were tested for embryonation by placing in a 3 in. watch-glass in a shallow tank, through which flowed continuously a stream of water maintained thermostatically at 24 ± 1° C. This method was unsuitable in experiments in which only very small numbers of eggs were produced, as some loss was inevitable in the subsequent handling. This difficulty was successfully overcome by the following technique. A trace of vaseline jelly was dabbed on to a 3 × 1 micro slide and rubbed vigorously with the finger until the vaseline was spread out as a thin invisible film. The eggs were concentrated, as far as possible, in about 2 c.c. of water at the bottom of the culture tube and picked up into a fine pipette. The drop of water containing the eggs was released carefully on to the slide surface and allowed to stand for about 10 min. The eggs sank in the drop and adhered to the glass. The slide was then placed, face downwards, on to two glass rods (acting as supports) in the trough, through which passed the stream of warmed water. The slide was removed at intervals for microscopic examination of the eggs.

EXPERIMENTAL RESULTS

The main results of culturing larvae at 40–30° C. are summarized in Table 1.

Table 1. *Results of culturing plerocercoids of* Schistocephalus *in horse serum* + 1 % *glucose at different temperatures*

Temperature (° C.)	Time to reach maturity (days)	Eggs produced	Eggs embryonated	Comments
40 (normal)	$1\frac{1}{2}$	+	+	Spermatogenesis normal (Figs. 1–7)
35	3	+	+	Spermatogenesis mainly normal, but a few abnormal spermatocyte morula present (Figs. 8, 10)
34	4	+	0	Eggs very abnormal in size and shape. Spermatogenesis very abnormal, with abnormal spermatocyte and spermatid morula (Figs. 8–15)
33	5	+	0	Ditto
30	Maturity never attained	0	0	Even after 29 days' cultivation, spermatogenesis did not develop beyond early spermatid morula, which were very abnormal (Figs. 8–10)

Egg production

40° C. At this, the normal temperature, eggs appeared in the uterus after approximately 36 hr. cultivation, but seldom were ejected through the uterine pore in less than 40 hr.

35° C. At this temperature, eggs have never been found in the uterus earlier than 70 hr. Such eggs did not appear to differ morphologically from normal eggs, but not more than 20 % showed embryonation on testing.

34° C. Eggs appeared in worms matured at this temperature on the fourth day of cultivation. Such eggs were very abnormal, showing great variation in size and shape. The shell was not uniform and showed marked thickenings in several places. These eggs failed completely to embryonate on testing.

33° C. At this temperature, worms produced a few, very abnormal, eggs on the fifth day of cultivation: such eggs failed to embryonate on testing.

30° C. Worms cultured at this temperature failed entirely to produce eggs, even after 29 days' cultivation.

Cytological abnormalities

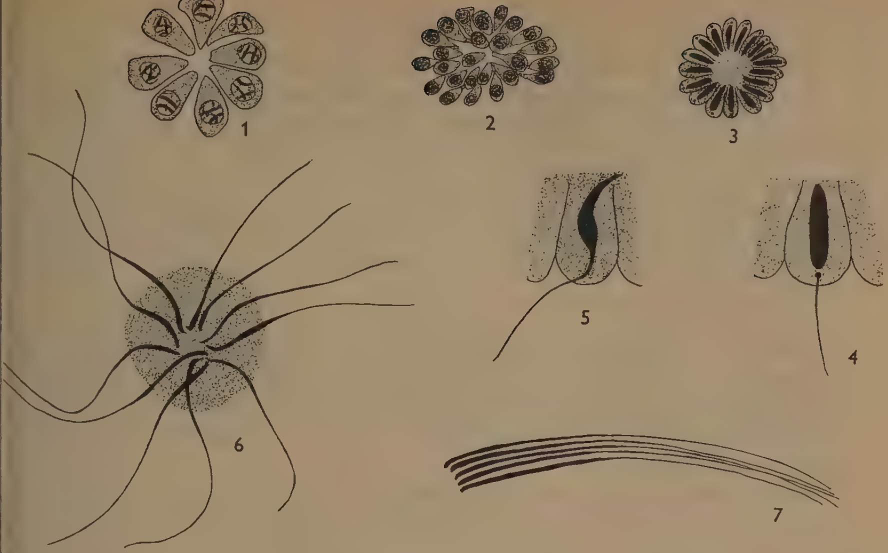
The histological and cytological changes occurring in larvae matured at the various temperatures were examined in considerable detail. The small size of the cells made observation difficult but, so far as could be ascertained, the only effect of reducing the temperature on the oogenesis, yolk and shell production, was to slow down these processes and no abnormalities were observed. On the other hand, very marked abnormalities appeared in spermatogenesis. Before describing these abnormalities, a brief account of the normal spermatogenesis must be given.

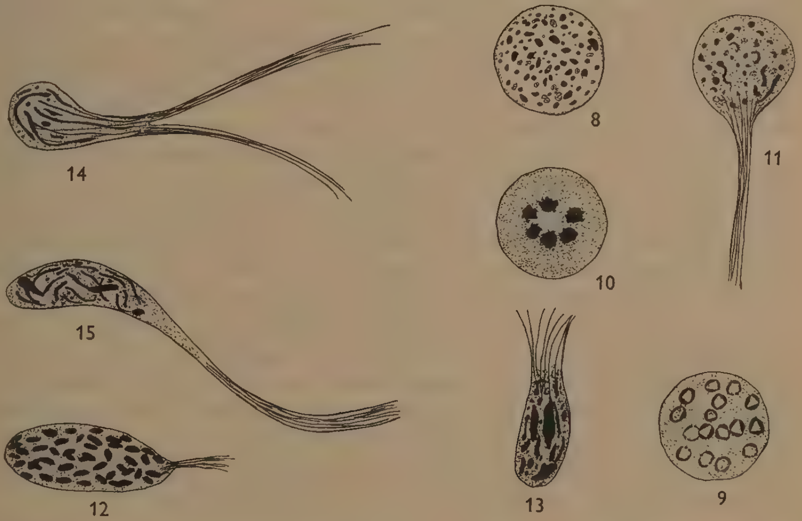
Normal spermatogenesis (i.e. at 40° C.). Spermatogenesis follows the typical pattern of *Platyhelminthia* in general. The spermatogonia, which occur at the periphery of the testis capsule, give rise, by repeated divisions, to a primary spermatocyte morula (Fig. 1) which undergoes meiosis, resulting in a secondary morula which gives rise to a spermatid morula. This last stage is readily recognizable by the elongated nuclei directed radially. At the periphery, centrioles appear from which tails grow out (Figs. 4, 5), and the nuclei undergo considerable lengthening, the head-piece becoming proportionately narrower. The extreme tip of the spermatozoan head appears coiled slightly at first, but gradually becomes almost straight. The fully formed spermatozoa break free from the residual protoplasm and appear as a bundle (Fig. 7), in which the spermatozoan heads all point in the same direction.

35° C. Although at this temperature eggs capable of embryonation are produced, the testes show some abnormal cells. These cells, termed in earlier papers (Smyth, 1946, 1950) 'giant polyploid cells', are spherical and their cytoplasm is markedly basophilic. Their nuclear material consists of a large number of chromosomes (Fig. 8) in various stages of division, being sometimes concentrated into definite nuclei (Fig. 10). The abnormal cells are rare, only one occurring in about every fifty testes capsules.

34° C. In worms matured at this temperature, 'giant polyploid cells' are common, one at least being found in almost every testis capsule; such cells do not differ significantly from those described above. In addition, very remarkable abnormal spermatid morula are very common. These (Figs. 11–15) have the form of a spindle-shaped oval or spherical cytoplasmic body, from which issue a number of spermatozoan tails. In the body can be distinguished chromatid material in various forms. In some the chromatin material is, in the main, granular (Fig. 11); in others, it forms well-defined spermatid nuclei (Fig. 12) similar to those occurring in normal spermatid morula (Fig. 3); in others, nuclei with the shape of a typical spermatozoan head can be identified (Figs. 14, 15), though considerable fusion of the chromatin material seems to occur; in others, irregular masses of chromatin are found.

33° C. The condition of the testis in worms matured at this temperature is, in the main, very similar to that described at 34° C., with the exception that normal spermatozoa are rare.





Figs. 1–7. Stages in normal spermatogenesis (i.e. at 40° C.). Fig. 1. Primary spermatocyte morula. Fig. 2. Secondary spermatocyte morula. Fig. 3. Spermatid morula with centrioles visible. Fig. 4. Enlarged spermatid with tail growing out from centriole. Fig. 5. Late spermatid showing curved head. Fig. 6. Mature morula with spermatozoa embedded in residual cytoplasm. Fig. 7. Bunch of mature spermatozoa.

Figs. 8–15. Various abnormal morula in testes of larvae cultured at temperatures of 30–35° C. Fig. 8 represents a typical spermatocyte morula with a large number of chromosomes (a so-called 'giant polyploid cell'). In Figs. 9 and 10, the chromatin is concentrated with definite nuclei, but cell walls are absent. Figs. 11–15 represent abnormal spermatid morula with pycnotic nuclei and tails growing out.

30° C. Normal spermatozoa were never found at this temperature. After 4 days' cultivation, the latest stage of spermatogenesis reached was the spermatocyte morula. By the eighth day, early spermatid morula appeared and about 25% of the testis capsules contained 'giant polyploid cells'. By the nineteenth day of cultivation, every testis capsule contained these cells and many had up to five in each capsule. A few abnormal early spermatid morula occurred, but sperm tails were never found and it is doubtful if, at this temperature, cells can even develop beyond the early spermatid stage for, even after 29 days' cultivation, no more advanced stages were observed.

DISCUSSION

It is clear from these results that normal maturation of pleroceroid larvae of *Schistocephalus* into sexually mature tapeworms producing eggs capable of embryonation cannot take place below 35° C. and that even at this temperature abnormalities of spermatogenesis appear. Below this temperature, spermatogenesis becomes exceedingly abnormal.

The occurrence of the peculiar 'giant polyploid' cells, containing large numbers of chromosomes and abnormal spermatid cells, is interpreted as being due to the degeneration and death of the cytoplasm with the survival, for some time at any rate, of the nuclear material. This explains all the types of abnormalities described at the various temperatures. The nuclei appear to be capable of considerable differentiation, even after the death of the cytoplasm, and sperm heads may even be formed, though at a later stage they become pycnotic and fuse in deeply staining masses.

It is evident from these results that, of the cells undergoing differentiation during maturation, those of the testes are the most sensitive to unfavourable conditions.

Similar abnormal cells have been described in *Schistocephalus* and *Ligula* matured *in vitro* at normal temperature, in poorly buffered media in which the acidic metabolic by-products rapidly produced a marked drop in pH (Smyth, 1949, 1950). These cells can now be identified as degenerating spermatocyte morula, as they do not differ in any way from those that occur in larvae cultured at 35° C. When first described (Smyth, 1946), their true nature was not recognized—since late abnormal spermatid morula with tails did not appear—though it was speculated that they were either abnormal cells or 'nurse' cells of some kind. There are, thus, two factors at least—subnormal incubation temperature and low pH, which can induce cellular degeneration in the testes, and probably several other factors could do likewise. Hence, investigation of the cytology of cultured worms forms a useful ancillary method for the assessment of the *in vitro* environment, the appearance of abnormal testes cells serving as an indication of unsuitable cultivation conditions.

SUMMARY

1. Plerocercoid larva of the cestode *Schistocephalus solidus* were cultured *in vitro* in horse serum + 1% glucose at the following temperatures: 35, 34, 33 and 30° C.; the temperature at which maturation normally takes place being 40° C.

2. Eggs capable of embryonation were never produced by larvae cultured at a temperature less than 35° C.

3. Below this temperature, spermatogenesis became markedly abnormal, resulting in the production of polyploid cells and degenerating spermatid morula.

4. No abnormalities in the vitellaria or ovaries were observed at these subnormal temperatures.

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THE ACTION OF THE PROVENTRICULUS OF THE WORKER HONEYBEE, *APIS MELLIFERA* L.

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(*Received* 13 *October* 1951)

(With Plate 13 and Seven Text-figures)

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INTRODUCTION

During the investigation of the infection of the ventriculus of the honeybee by *Nosema apis*, information was needed of the passage of pollen grains through the intestine. Some of the work of Whitcomb & Wilson (1929) was repeated for this purpose and results contradictory to those of these workers were obtained, particularly those concerning the action of the proventriculus.

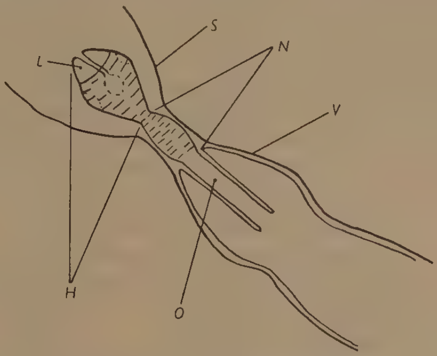
REVIEW OF LITERATURE

The mode of action of the proventriculus has been a subject of some controversy.

Trappmann (1923) gives a fairly detailed account of the histology of the organ. His brief account of its action simply states, however, that the four lips, by means of snapping and sucking movements, lead food back from the crop to the mid-gut. He also states that the proventriculus prevents nectar from flowing back from the crop to the ventriculus in the foraging bee and that it prevents the ventricular contents from being regurgitated into the crop. The latter action is effected by the oesophageal invagination (Text-fig. 1). The following discussion refers only to the part of the proventriculus within the honey stomach (Text-fig. 1—'Head') and the part connecting the honey stomach with the ventriculus ('Neck').

Trappmann's idea that the band of circular muscle around the neck prevents the evagination of the head into the ventriculus indicates that the true action of the proventriculus had escaped his observations.

Snodgrass (1925) does not agree that the proventriculus is a filtering organ. 'It has often been supposed that the proventricular mouth enables the worker to pick out pollen grains from the nectar in the honey stomach and pass them on into the stomach, leaving the nectar to be carried into the hive and stored in a cell. But the worker does not eat pollen while she is engaged in collecting nectar, and if pollen is found in the honey stomach it is only such as was contained in the honey taken from the cell.' This answer simply evades the question. He also states that the movements of the lips 'suggest their picking imaginary pollen grains out of nectar, but the action is probably merely the ordinary process by means of which the proventriculus passes to the ventriculus any kind of food in the honey stomach'. He does not indicate what this 'ordinary process' really is.



Text-fig. 1. Diagram of proventriculus. *L*, lip; *S*, honey stomach; *N*, 'Neck'; *V*, ventriculus; *O*, oesophageal invagination; *H*, 'Head'.

Whitcomb & Wilson (1929) show that the proventriculus is capable of separating pollen grains from fluid in the crop. However, their description of the whole organ thrusting forward into the crop and then withdrawing, combing out the pollen, which is immediately passed back into the ventriculus is undoubtedly erroneous, as will be seen later. Their description of pollen collecting locally around the lips of the proventriculus before being picked out of the crop may have been that of an artifact described later.

Imms (1934) states that the proventriculus serves to pump food from the crop to the ventriculus. This action, as will be seen later, takes place, but it is only part of the function. He speaks of an X-shaped lumen which, guarded by four lips, leads from the crop into the ventriculus. This interpretation of the lumen could be suggested by a study of transverse sections of the proventriculus (Pl. 13, fig. 2), but it is not the permanent shape.

Zander (1922) and Metzer (1910) correctly described the pumping action of the proventriculus and stated that the fringe of hairs on the edge of the lips (Text-fig. 7) acts as a rake, probably allowing nectar to *pass back to the crop* but holding the pollen grains in the cavity of the proventriculus. Their hypothesis is quite correct but they did not develop the idea at all, in spite of the detail which they observed. They were perhaps thinking of pollen being cleared from the nectar gathered by foragers which may have led Snodgrass to deny such filtering activity on the grounds quoted above. Zander describes the lips as being the means whereby the forager prevents nectar from entering the midgut and says that the sucking action of the proventriculus allows the contents of the crop to pass back to the ventriculus in a steady stream. Both these statements are incorrect.

Hejtmanek (1933) mentions that pollen is passed as a bolus by the proventriculus from the honey stomach into the ventriculus. However, his observations were based on the examination of the alimentary canal after it had been extracted from the bee, and so the action of the proventriculus was not seen. He simply states that the chitinous lips form the pollen into clumps.

His statement that the pollen remains in even suspension within the honey stomach and that pollen passes to the hinder end of the ventriculus within a few minutes were confirmed by the experiments described below.

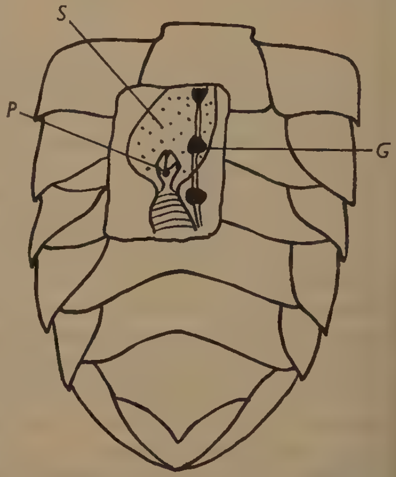
He denies that the concentration and volume of pollen suspension has any effect upon the speed of passage of pollen through the alimentary canal. This is only true for the passage of pollen through the ventriculus and hind-gut, but is not true for its passage through the honey stomach and proventriculus.

MATERIAL AND METHODS

Hazel pollen and pollen taken from the pollen baskets of bees were stained bright red with magenta red, suspended in clear syrup and fed to starved individual worker bees from a capillary pipette. For observations on the passage of pollen down the midgut the whole gut was pulled out of the abdomen of the anaesthetized bee by cutting off the head and pulling out the last abdominal segment with forceps. Careful slitting of the ventriculus with fine needles, or dehydration and clearing in cedar-wood oil, showed the state of the pollen.

For observation of the action of the proventriculus the starved bee was anaesthetized with chloroform or ether and secured ventral side uppermost to a soft wax block.

A window was carefully cut out of the sternites (Text-fig. 2) with a sharp razor. The film of fat body was carefully pushed aside with blunt needles, and the proventriculus was then easily visible when illuminated directly with a focussing lamp.



Text-fig. 2. Ventral view of abdomen showing window dissection; *S*, honey stomach; *G*, ganglion; *P*, proventriculus.

If necessary it could be brought nearer to the surface by hooking round the neck with a blunt, bent, entomological pin. Care was taken not to injure the ventral nerve cord and ganglia. These would usually slip over to one side to allow clear vision.

Occasionally the window was moistened with a drop of saline (0·75 % NaCl, 0·02 % KCl, 0·02 % $CaCl_2$ buffered to pH 6·7 with Na_2HPO_4 and HCl). If the drop of saline was large enough, the illumination of the proventriculus was aided by its additional focusing effect.

THE PASSAGE OF POLLEN DOWN THE VENTRICULUS

(*a*) *Brood-rearing bees*

Bees were collected from the top of the combs during March and April when brood-rearing was in progress and extensive foraging had not yet begun.

The bees were starved for 1 or 2 hr. in an incubator at 33° C. They were then fed with a drop of pollen suspension by holding them upside down by the wings and allowing a drop to fall on to their mouth-parts from the pipette. After feeding they were kept in separate containers in the incubator for definite periods of time, after which their alimentary canals were pulled out and examined.

Table 1. *Rate of progress of pollen down alimentary canal*

Bee	Period after feeding	Position of pollen package
		(*a*) Brood-rearing bees
1	5 min.	Half-way down ventriculus
2	45 min.	Half-way down ventriculus
3	1 hr.	Posterior end of ventriculus
4	3·5 hr.	Three-quarters way down ventriculus
5	3·5 hr.	Posterior end of ventriculus
6	5·5 hr.	Posterior end of ventriculus
7	14 hr.	Posterior end of ventriculus
		(*b*) Nectar-gathering bees
1	1 hr.	Posterior end of ventriculus
2	2·5 hr.	Entering hindgut
3	3 hr.	One-third way down hindgut
4	4 hr.	Three-quarters way down hindgut
5	6 hr.	In rectum

The pollen was usually gathered in the ventriculus in packages surrounded by a tough skin of peritrophic membranes, as described by Whitcomb & Wilson (1929). However, the number of packages and their positions in the ventriculus varied from bee to bee.

In some cases the package would be near the hindgut after about five minutes. In others it would only reach the hindgut after 2 or 3 hr. In one bee, dissected after 14 hr., the pollen was still in the ventriculus even though the rectum was relatively empty for the time of year. No red pollen at all was present in the hindgut or rectum. Many other bees were observed to retain the pollen in the ventriculus for many hours (Table 1 *a*).

On several occasions it was seen that the pollen packages at the hind end of the ventriculus were composed of two clearly demarcated halves. The anterior half was formed of red pollen and the posterior half was unstained. The unstained pollen was obviously that which had been eaten in the hive. (Stained pollen never lost the dye, even when it finally reached the rectum and had been there for a day or longer.) This indicated that the pollen had moved relative to the encircling peritrophic membranes and that the membranes move down the gut at a much slower pace.

(*b*) *Foraging bees*

Later, in May, foraging bees were taken from the flowers where they were working and experiments similar to the above were repeated.

The red pollen in these experiments reached the rectum fairly regularly and in a far shorter space of time (Table 1 *b*). The bees were fed the weak suspension of pollen (see § (*c*)).

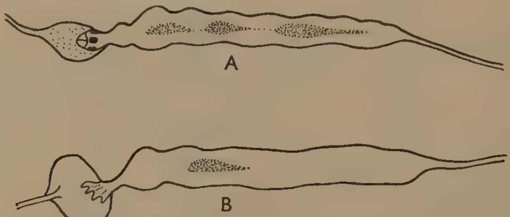
Very few scattered grains of unstained pollen were present and these were principally in the rectum.

(*c*) *Effect of concentration of pollen*

Bees were then fed with different concentrations of pollen, one concentration being much stronger than that used previously.

The more concentrated pollen produced a chain of packages in the ventriculus, some of which reached the hind end of the ventriculus after 4 or 5 min. (Text-fig. 3 *a*).

The weaker concentration of pollen generally produced one packet about half-way down the ventriculus after about 20 min. (Text-fig. 3 *b*).



Text-fig. 3. Pollen packets in the ventriculus. A. After feeding with concentrated pollen. B. After feeding with dilute pollen.

The bees fed on the weaker suspension had a clear or nearly clear honey stomach after about 20 min.

It seems, therefore, that the bee is capable of filtering pollen from the fluid in the crop. It may have been a weak suspension of pollen which Whitcomb & Wilson (1929) used, requiring but one packet of pollen to clear the crop.

Whitcomb & Wilson (1929) state that the peritrophic membranes in the ventriculus contract round the pollen grains to form the package of pollen. It seemed strange that this action, if it occurs, could form a chain of practically discrete packages. The only organ capable of producing such packages seemed to be the proventriculus itself. When weak suspensions of pollen were fed, it presumably gathered the whole amount of pollen together and then passed it back to the ventriculus. When strong pollen suspensions were fed, a package was passed back long before the crop was clear of pollen, several packages being necessary to achieve this end.

THE ACTION OF THE PROVENTRICULUS

If the gut is pulled out of the abdomen of the bee and laid on a warm slide, the proventriculus will continue to move its lips for some minutes. However, the action is usually weak and fitful.

If, however, a window is dissected out of the abdomen of the bee, as described above, the bee will live for many hours if kept warm and the window kept moist with buffered saline.

The action of the proventriculus is vigorous and regular for periods up to 30 min. or an hour when the dissection is good with minimum damage to nerve cord, tracheae, air sacs, etc. It will probably live for much greater periods, but this has not been necessary for the purpose of the following experiments.

When the bee was fed with pollen suspension the pollen was seen passing into the crop within a second or two and the crop distended rapidly. Pollen became distributed evenly throughout the crop and remained so disposed until only a few grains were left. The crop was seen to be continually writhing and pulsating vigorously. This action was so strong that the pollen grains could be seen moving about inside, and the action obviously kept the crop contents well stirred and the pollen grains evenly distributed. The proventriculus itself never moves as a whole relative to the honey stomach, although it may be moved to some extent by the powerful activity of the crop.

This activity of the crop was rarely seen when the alimentary canal was pulled out of the abdomen and was quickly destroyed if the window dissection was done badly. The organ is obviously unable to exert much, if any, pressure, but a contraction of the branching and anastomosing muscle fibres of the wall (Wigglesworth, 1947) in some areas and a corresponding relaxation in others creates no pressure resistance but provides an admirable stirring movement.

Pollen was seen to accumulate rapidly in the proventriculus. At first this accumulation took place in four 'cheeks' or pouches, and these rapidly filled with tightly packed pollen (Pl. 13, figs. 5, 6). These pouches seemed to separate and then to collapse together, with a gradually decreasing amplitude as the proventriculus filled with pollen. It appeared that the proventriculus was filling and emptying. However, the emptying was not into the ventriculus but back into the crop. (When syrup, which had been stained deeply with gentian violet, was fed to the bee, the filling and emptying of the crop could be seen quite clearly, but the dark ball of syrup in the proventriculus disappeared when the organ emptied and usually did not pass back to the ventriculus. Occasionally, however, a bolus of dark syrup moved down the neck into the ventriculus. On one occasion the proventriculus developed the shorter stroke as when straining pollen, but in this case it was found to be packed with crystals of gentian violet which had not dissolved completely in the syrup.)

After a while the proventriculus was packed with pollen, with a solid core as well as the full pouches, and eventually the whole mass passed as a bolus down the neck into the ventriculus, leaving but one or two grains behind in the pouches (Pl. 13).

The movements of the lips during these processes were asynchronous. They snapped open and closed very rapidly but individually. They appeared to be letting pollen suspension into the proventriculus during the expansion process by these movements and to be guarding against the ejection of grains back into the crop.

No accumulation of pollen in the crop near the lips of the proventriculus, as described by Whitcomb & Wilson (1929), was observed. However, in one or two cases when the alimentary canal was pulled out of the abdomen and laid on a slide, a proventriculus full of pollen would collapse and discharge its contents into the crop giving a local cloud of pollen grains. Sometimes the proventriculus would continue repeatedly to take in and expel the cloud of pollen as though the guarding action of the lips had been deranged. Rough handling of the gut during its extraction often caused this to happen, and the proventriculus quickly ceased to perform any significant action. Probably some damage had been done to the organ. In any case the action was weak and spasmodic and was undoubtedly an artifact. It was never observed in the 'Window dissections'.

Sometimes, if the bees had been starved for long periods (4 or 5 hr.), the first action of the proventriculus would be to pass back two or three boluses at once without any filtering action and then to start the normal filtering. This occurred whether the syrup contained pollen or not.

When pollen and syrup was taken in this way the pollen remained in a dispersed condition in the ventriculus and showed no signs of being collected into a packet.

THE EFFECT OF CONCENTRATION OF POLLEN ON THE ACTIVITY OF THE PROVENTRICULUS

(*a*) *Qualitative experiments*

Windows were dissected out of the abdomens and the bees were fed:

(1) Syrup heavily stained with gentian violet.
(2) Pollen suspension with 1 volume of pollen to 15 volumes syrup.
(3) Pollen suspension with 1 volume of pollen to 7 volumes syrup.

With stained syrup, nineteen boluses were passed to the ventriculus. The first bolus was passed after 3 min. and the rest followed at intervals varying between 15 and 40 sec. with an average interval of 21 sec.

With the weaker pollen suspension four boluses of pollen were observed with intervals, in seconds, of 380, 380, 200, 140.

Since the time intervals for the first two boluses occupied nearly 13 min. heat was applied by means of a hot spatula held reasonably close to the window. The activity was increased and the further two boluses were passed after much shorter intervals. (Apart from this particular application of heat all the experiments were carried out during the same afternoon at room temperature (about 20° C.).)

With the stronger suspension the intervals were, in seconds, 90, 50, 230, 60, 150. Thus it seems that boluses are passed back more quickly, the stronger the suspension of pollen in the crop. This is to be expected, assuming a uniform activity of the proventriculus at a fixed temperature and a uniform dispersion of pollen in the crop.

To try and gain more controlled conditions unaffected by unknown hazards of the dissection the bees were fed quantitatively and killed for examination after definite intervals of time. Bees were collected from the top of the combs and anaesthetized with ether. This caused vomiting of the crop contents. They were allowed to recover in the incubator at 33° C. and were kept there without food for 2 hr. After this time they begin to weaken very quickly and the crop was quite empty.

They were then stuck on glass rods with a piece of soft wax by the dorsal side of the thorax.

Each bee was fed 0·01 ml. of pollen suspension from a calibrated capillary pipette, and after a definite time interval in the incubator was killed, and the whole of its alimentary canal extracted.

(1) Six bees fed pollen suspension with 1 volume pollen to 7 volumes syrup. They were killed at 3 min. intervals. The crops were all about the same size but the gradation of strength of pollen suspension was obvious.

(2) (*a*) Six bees fed a suspension with 1 volume pollen to 3 volumes syrup killed over the same time intervals as in Exp. 1. They showed no obvious difference in pollen concentration. The crops were all the same size.

(*b*) Four bees were fed the 1 : 3 suspension and killed over intervals shown below:

(i) 30 min.: heavy concentration of pollen in crop.
(ii) 45 min.: concentration obviously lower.
(iii) 70 min.: one or two grains only, in the crop.
(iv) 85 min.: clear fluid in crop. This crop was markedly less in size than all the others, including Exps. 1 and 2(*a*).

(*b*) *Quantitative experiments*

Counts were then made of pollen grains in the honey stomach by means of a haemocytometer. The crops were burst on the calibrated grid and stirred gently with a wooden splint to ensure even distribution.

Each bee was fed 0·01 ml. of suspension containing 6500 grains/cu.mm. The results are given in Table 2. The figures under 'Minutes after feeding' refer to pollen concentration in grains per cu.mm.

Table 2. *Pollen concentration in the honey stomach*

Series	Minutes after feeding												
	1	5	7	10	12	15	17	20	23	25	27	30	35
1	—	500	—	420	—	—	—	—	—	260	—	100	0
2	1000	780	380	440	380	—	—	—	—	260	—	100	—
3	4000	—	3250	—	—	—	—	1350	—	—	—	—	—
4	—	—	5280	—	—	—	2500	—	3250	—	2400	—	—
5	—	2720	—	600	—	2160	—	130	—	30	—	40	0

Considerable variation in the performance of individual bees is still obvious, but in general it may be said that the concentration of pollen grains is reduced at a remarkable rate over the first few minutes, and then this rate of reduction is less marked when the concentration of pollen in the crop falls to a low level.

A more reliable method of starving the bees was developed by taking bees as they emerged from the hive entrance and starving them for 1 hr. These bees then invariably had an empty honey stomach.

The method of filtration by the proventriculus, together with the maintenance of the even distribution of pollen grains within the honey stomach by the vigorous movements of the walls of the latter, suggested that the concentration of pollen grains would follow a logarithmic curve when plotted against time.

There were four experiments in which individual bees were fed:

(1) 0·02 ml. suspension containing about 190,000 pollen grains/ml.

(2) 0·02 ml. suspension containing about 20,000,000 spores of *Nosema apis*/ml. (Spores were obtained by grinding up the ventriculi of infected bees in a little water and filtering the fluid through a small, loosely packed plug of cotton wool. The spores passed through the filter leaving all other solid matter behind. The spores were then concentrated by centrifuging the filtrate.)

(3) 0·02 ml. suspension containing 1,450,000 pollen grains/ml.

(4) 0·04 ml. suspension containing 1,450,000 pollen grains/ml. The bees were then kept in small cages in an incubator at 34° C. for definite periods of time and the concentrations of particles within the honey stomach were determined as before.

The results are shown graphically in Text-fig. 4 A, B, C and D.

The curves were derived statistically on the assumption that they were exponential of the type $y=be^{-at}$, where y=pollen concentration at any particular time t, b=initial concentration of pollen, $e=2\cdot71828$, $a=1/T$, where T is the time constant.

The values of b and a were derived from the normal equations

$$5\cdot3020\Sigma y_i^2 \log b - 2\cdot3026\Sigma y_i^2\, t_i\, a = 5\cdot3020\Sigma y_i^2 \log y_i,$$

and

$$5\cdot3020\Sigma y_i^2\, t_i \log b - 2\cdot3026\Sigma y_i^2\, t_i^2\, a = 5\cdot3020\Sigma y_i^2\, t_i \log y_i,$$

and the variance of a was derived from the equations

$$\sigma^2 = \frac{5\cdot3020}{n-2}\left[\Sigma y_i^2(\log y_i)^2 - \log b\Sigma y_i^2 \log y_i + 0\cdot43429a\Sigma y_i^2\, t_i \log y_i\right],$$

$$\Delta = (5\cdot3020\Sigma y_i^2)(2\cdot3026\Sigma y_i^2\, t_i^2) - (5\cdot3020\Sigma y_i^2\, t_i)(2\cdot3026\Sigma y_i^2\, t_i),$$

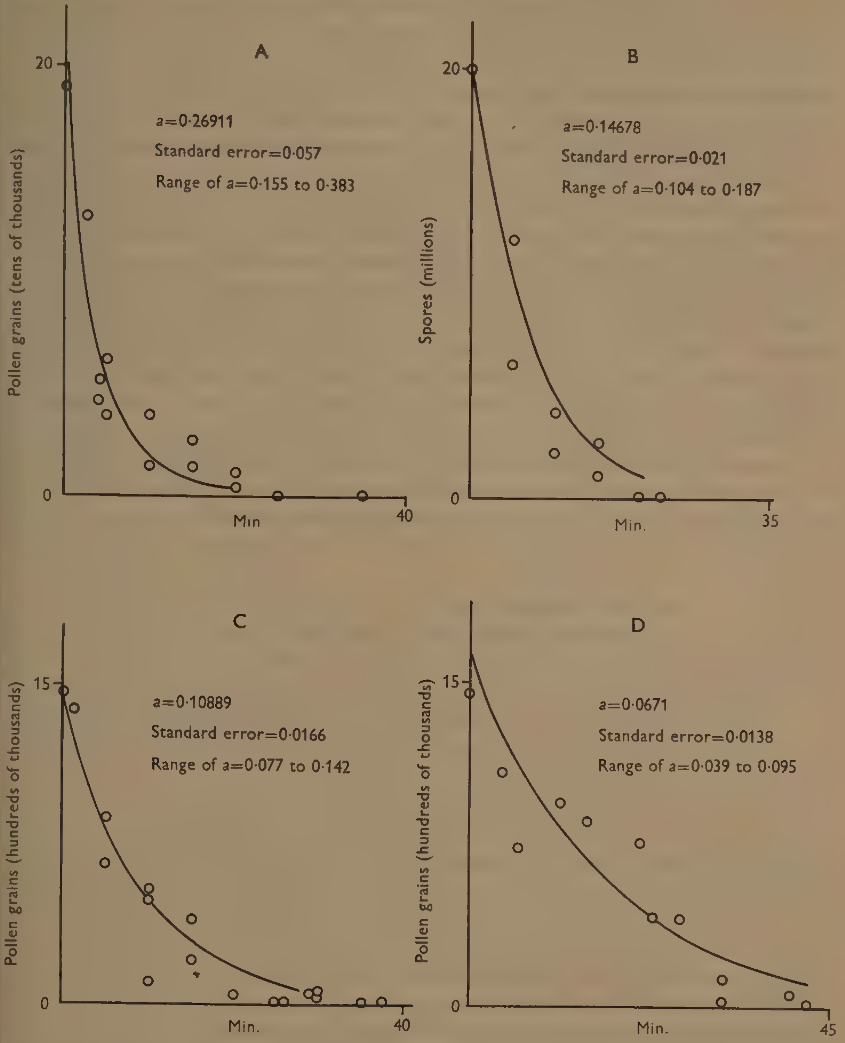
$$\text{Variance of } a = \frac{2\cdot3026\,\sigma^2(5\cdot3020\Sigma\, y_i^2)}{\Delta}.$$

The 5 % fiducial limits of a, ($a \pm 2\sigma_a$), "define the range of a".

It is seen that Text-fig. 4A is significantly different from Text-fig. 4C and D and Text-fig. 4B is significantly different from Text-fig. 4D.

The high value of T in Text-fig. 4D is due to the high volume of fluid, since the proventriculus has a fixed maximum volume and so can only filter a certain fixed volume per unit time. It will therefore take longer to clear a crop distended with pollen suspension compared with a crop containing only a small volume of fluid even if the concentration of the pollen in each crop is the same.

The low value of T in Text-fig. 4B (spores) is due to the small size of the spore of *Nosema apis*. The spore is only about 6 by 3 μ, whereas the pollen grains were between about 16 and 32 μ. The spore therefore is considerably smaller in volume than the average pollen grain, and the proventriculus is able to maintain an expansion and contraction of high amplitude, since the spores are packed away into the pouches. This means that the high rate of filtration can continue over a long period of time with only a few boluses to pass back into the crop.



Text-fig. 4.

Thus the time constant of Text-fig. 4B is lower than that of Text-fig. 4C, in spite of the higher concentration of particles represented by Text-fig. 4B.

Text-fig. 4A represents a pollen concentration whose total volume of solid matter was probably less than that in the spore suspension, and so the time constant of Text-fig. 4A is less than that of Text-fig. 4B.

Text-fig. 4C also shows the effect of the choking of the proventriculus. The volume was the same as in Text-fig. 4A, but the volume of solid matter was higher and so the time constant is higher than the latter.

It should be noted that if the proventriculus remained at a constant efficiency, regardless of the amount of solid matter within its lumen, then the rate of fall of concentration of any suspension of particles, however large, or small the concentration or size of particle, would be the same, provided the honey stomach contained the same volume in all cases.

The significant difference between the time constant of Text-fig. 4A and C is therefore due to the choking effect of the higher volume of solid matter on the proventriculus and the larger number of boluses which had to be passed, and not simply due to the larger concentration of particles, represented by Text-fig. 4C, which had to be filtered off. As described above, and as suggested by the short time constant of Text-fig. 4B, the actual concentration of particles is not the deciding factor. Indeed, Text-fig. 4C represents the filtration of more pollen grains in actual numbers per unit time than that represented in Text-fig. 4A, but the real efficiency of filtration was less.

The complication of the proventriculus being filled by solid matter, and so being reduced in efficiency, affects the curves, which cannot therefore be strictly exponential in character. They will be less steep than exponential curves at first and will gradually approach a curve of exponential nature because the efficiency of the proventriculus becomes less affected by the remaining smaller volume of solid matter. Also the proventriculus gradually becomes relatively more efficient, especially where the volume of solid matter was initially great compared with the total volume, because the total volume is gradually reduced by the removal of the solid matter. The curves also all fall to zero values in actual fact, whereas the derived curves do not. This means of course that the lower range of a, as derived statistically, is too low in all four cases. This may mean that all four curves really are different from each other (cf. lowest values of a in all four curves).

It may be said therefore that with the same volume of fluid and particles of the same size, but different concentrations, the lower concentration will be filtered more efficiently than the higher concentration (cf. Text-fig. 4A, C).

Smaller particles will be filtered more efficiently than larger ones (Text-fig. 4B).

With different volumes of the same concentration of particles of the same size the higher volume will be filtered more slowly (Text-fig. 4D).

There is, of course, no discrimination by the proventriculus of different-sized particles contained within the same suspension.

Maurizio (1949) found that the pollen in the honey stomach was reduced in concentration over a period of time and found that the rate of reduction was highest during the first minute—the concentration falling to half that which was fed. Thereafter the concentration fell only slowly. She could not account for this initial rapid fall in concentration because no pollen could be found within the ventriculus in more than half the bees examined. However, the bees had been kept in cages prior to the experiment and were starved for only half an hour. When bees are kept in cages they always keep their honey stomachs very full indeed, and 2 or 3 hr. (even at 34° C.) must elapse before the honey stomach is empty. Consequently her bees must have contained an appreciable amount of syrup when they were fed the pollen suspension and so the latter being diluted immediately it entered the honey stomach would account for the initial rapid reduction in concentration without any pollen leaving the honey stomach.

Maurizio found that the concentration was reduced by one-half or one-third during the first 16–20 min. discounting the initial reduction during the first minute or two. However, the honey stomach of the bees probably contained a greater volume than the 0·03 ml. which they were fed, and so the time constant was quite large, giving the more gentle slope of her curves relative to those described in this paper.

Maurizio was trying to find whether the concentration of pollen grains or their size had any effect on the rate of fall of concentration within the honey stomach, and, from conflicting statistical analyses, came to the conclusion that neither had any effect. For the practical purpose of honey analysis, it may be that the relatively slight variations in size of pollen grains, which the bee collects accidently whilst gathering nectar, produce little significant effect upon the pollen content of the fluid deposited in the cell of the comb, but the volume of nectar carried back to the hive will determine the quantity of pollen which actually arrives.

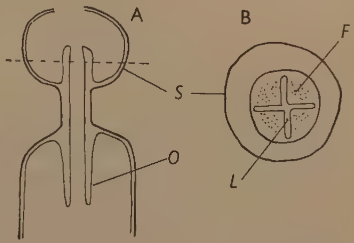
Only a study of the structure of the proventriculus and its action can give a clear picture of all these phenomena.

THE STRUCTURE OF THE PROVENTRICULUS AND THE MECHANISM OF FILTRATION

For the purpose of description the proventriculus may be regarded as a thickened inflected part of the crop where the latter leads to the ventriculus (Text-fig. 5). It has four folds which are continuations of the four major folds of the crop (Snodgrass, 1935).

Both crop and proventriculus have an outer layer of transverse musculature and an inner layer of longitudinal musculature. The longitudinal musculature of the proventriculus is powerful and by contraction causes the lumen of the organ to enlarge.

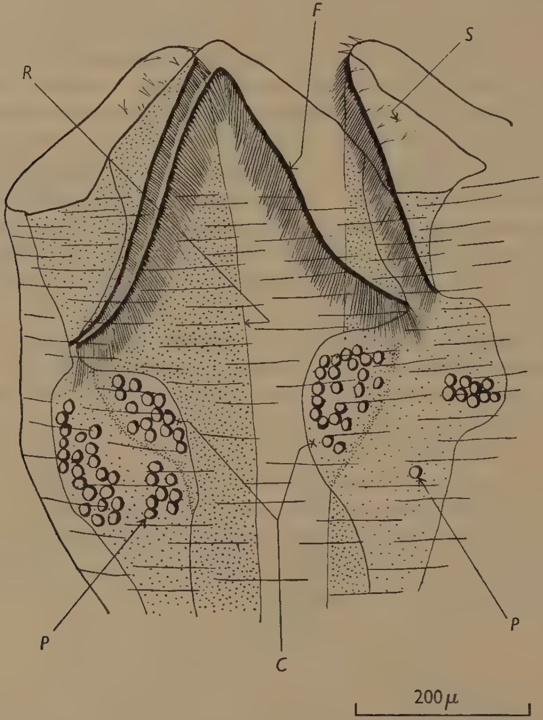
The 'lips' are extensions of the folds beyond the encircling band of transverse musculature, and in addition to the longitudinal muscles have short muscles connecting their tips to the outer wall. The contraction of the latter would cause the lips to open.



Text-fig. 5. Diagram of proventriculus. A. Longitudinal section. B. Transverse section. *L*, lumen; *F*, fold or ridge of longitudinal musculature; *S*, honey stomach; *O*, oesophageal invagination.

The circular muscles are quite powerful but are somewhat thinner over the pouches (Pl. 13, fig. 3). This probably allows the pouches to expand as they fill with pollen. The chitin over the pouches seems to be endocuticle with its layer of epicuticle (Pl. 13, fig. 4), and so, without the exocuticle, will be quite elastic (Wigglesworth, 1947). The circular muscles round the neck are thicker than the rest and probably act as the sphincter described below.

The combs of hairs on the edge of the folds appear to be capable of being folded in upon the surface of the fold or to be opened away from the surface. Perhaps the contraction of the longitudinal muscles exerts a pressure, as they swell, against the sides of the folds, which are less heavily chitinized than their inner surface, and thereby close up the hairs against the surface. This will give unrestricted entry to pollen grains rushing in to the expanding lumen of the proventriculus.

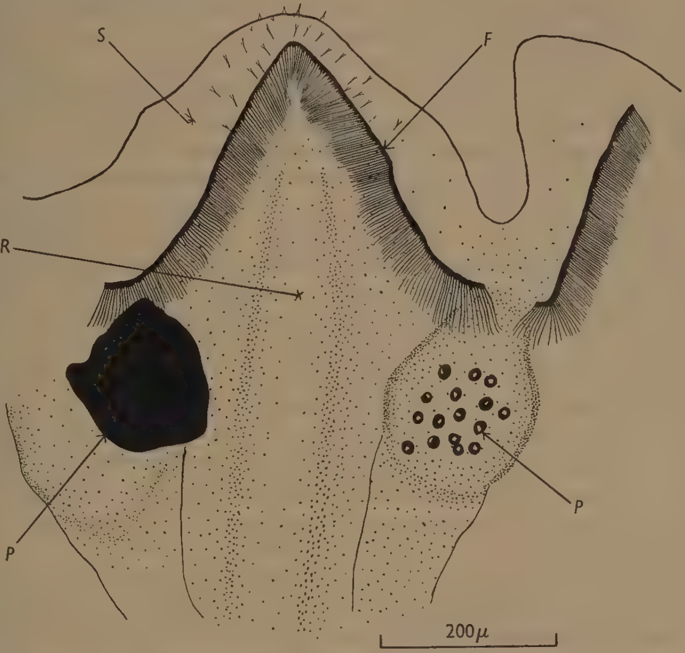


Text-fig. 6. Outer view of proventriculus. Specimen fixed in Bouin, dehydrated, cleared and mounted whole. The fourth lip and the two associated pouches behind are omitted to avoid confusion. *F*, fringe of hairs; *R*, internal ridge of longitudinal muscle; *P*, pollen grains in the pouches; *C*, crypts of the pouches between the encircling band of circular musculature and the internal longitudinal musculature; *S*, short spines.

When the longitudinal muscles relax, the hairs will open—perhaps automatically by the release of tension on the strained chitin. When the proventriculus is examined by carefully prising the lips apart with needles, the hairs are seen to be raised from the surface of the fold and are pointing inwards to the lumen.

The contraction of the circular muscles will cause the expulsion of the fluid contents of the proventriculus back into the crop, the thicker sphincter layer preventing entry into the ventriculus.

Pollen grains will be sieved off by the comb and forced into the pouches as the folds collapse upon each other. Repeated intake and expulsion of contents in this manner will gradually cause a mass of pollen grains to accumulate in each pouch (Text-figs. 6, 7; Pl. 13, fig. 6).



Text-fig. 7. Inner view of proventriculus. Showing one fold and associated parts with the two adjoining pouches. Proventriculus slit and opened out after immersion in Canada Balsam. The pollen mass to the left was dislodged from the pouch. Lettering as in Text-fig. 5.

Finally, a large mass of pollen grains is collected, and then the contraction of the circular muscles forces this large bulk against the hairs of the combs. Perhaps this back pressure on the sphincter causes it to open suddenly. A bolus of pollen passes through the neck into the ventriculus leaving but a few grains of pollen behind in the collapsed pouches (Pl. 13, fig. 5).

Whitcomb & Wilson (1929) show that the shells of the pollen grains are not broken at any stage, yet their contents are completely digested in the ventriculus.

The structure and operation of the proventriculus in the drone appear to be exactly like those of the worker bee.

The structure of the proventriculus in the queen bee, from an examination of preserved material, appears to be exactly like that of the worker.

DISCUSSION

Beutler & Opfinger (1949) and others have shown that pollen feeding influences the length of life of the adult bee. They point out that it is only the young bee (10 days old or less) whose life is significantly increased by pollen feeding.

Lotmar (1939) showed that the length of the ventriculus of the brood-rearing bee (about 11–12 mm.) was appreciably longer, on the average, than the length of the ventriculus of other types of bee. She also showed (1951) that the dry weight (*less* the whole alimentary canal) of bees gradually increased up to 11 days and then began to decrease slowly in the foraging bee.

I have shown that the foraging bee passes pollen down the gut to the rectum at a greater rate than the bees taken from brood-rearing colonies and from the top of the combs (principally brood-rearing and comb-building bees).

Perhaps the young bee has a more active ventriculus than the older bee, and perhaps pollen digestion is carried out to a more significant degree in the younger bee.

Lotmar (1939) showed that pollen consumption, by the colony, passes through a maximum in September or October when brood-rearing has practically ceased. The protein is stored in the fat bodies principally in the abdomen, and only a relatively small proportion of this protein is used up during the winter (the protein was expressed as nitrogen content estimated by Kjeldahl analyses). Most of this protein disappears in the early spring when brood-rearing starts again. So it appears that the young bees in autumn assimilate protein, which is stored in the fat bodies, since it is not now expended as brood food, but the older, over-wintered bee could not take part in brood-rearing if it ate the pollen immediately before brood-rearing commenced because its powers of digesting protein may be somewhat diminished. It is noteworthy that the over-wintering bee has a shorter ventriculus than the young bee (about 8–9 mm.).

The caged bee, captive for 2 or 3 weeks and fed on syrup has the shortest gut (about 7–8 mm.), and Lotmar remarks on the epithelial cells of the ventriculus of these bees being 'somewhat curiously short and reduced'. All these facts suggest a diminished activity of the ventriculus in the older bee and, perhaps, atrophy of the organ.

Consideration of the source of invertase within the worker bee suggests a possible function of the proventriculus which may be of importance to the physiology of digestion. Evenius (1926) refers to the 'Speicheldrüsen' as the source of invertase, but used the whole head for his experiments and so his glands were really mostly pharyngeal. Ingelscent (1940) found invertase only in the thoracic salivary glands and none in the pharyngeals. Kosmin & Komarow (1932) and others (Wigglesworth, 1947) refer to the pharyngeals as the source. However, it is likely that sucrose is digested principally in the crop by invertase from these glands. Langer (1909) indicated by serological technique that the invertase in honey was identical with that found in the glands.

Pavlowsky & Zarin (1922) and Kosmin & Komarow (1932) found invertase in the ventriculus, but they used the whole organ, including contents for analyses, and their results therefore do not preclude the supply of this enzyme from a more anteriorly situated source. Evenius states that the concentration of invertase in the ventriculus decreases down its length, suggesting its supply from the crop and its gradual dilution or destruction within the ventriculus.

It may be that the ventriculus of the bee is an organ primarily producing proteolytic enzymes and is not greatly concerned with the digestion of sucrose.

In any case, the proventriculus filters off the pollen as compact masses and leaves the syrup or honey behind. This alone probably facilitates the digestion of pollen, since the proteolytic enzymes are not diluted by an excess of fluid. The proventriculus therefore serves the purpose of dividing the two principal items of food for separate treatment. (Butler (1949) indicates the doubts expressed of the capability of the bee to digest polysaccharides and lipoids.) A few experiments on the effect of honey upon the proteolytic activity of the ventriculus showed that this separation of the two food constituents must be advantageous to their efficient digestion.

Ventriculi of pollen-eating bees were ground up in measured quantities of the buffered saline described above, and drops of the fluid, serially diluted with the saline, were incubated on the gelatin emulsion on strips of photographic plate. The strips were placed in Petri dishes lined with wet filter-paper and were incubated at 34° C. The gelatin was completely digested into water-soluble products after 1 hr., leaving a clear hole when the plate was washed in cold water, and only dilutions equivalent to one ventriculus in 2 or more ml. of buffer showed incomplete digestion.

Parallel experiments, using portions of the same stock of ground-up ventriculi but using fresh honey as the diluting fluid, showed that concentrations equivalent to one ventriculus in 0·5 ml. or less of a solution equivalent to 25% honey, were needed to effect complete digestion of the gelatin. Both honey buffered to pH 6·7 and pure honey gave the same effect.

The effect of the honey is probably a competitive action by the sugar molecules with the proteolytic enzymes for water, as shown by Nelson & Schubert (1928).

Besides avoiding dilution of the enzyme therefore this efficient separation of pollen must reduce the inhibitory action of honey upon proteolytic activity to a considerable extent.

SUMMARY

1. The proventriculus of the worker honeybee is an organ which effects a highly efficient separation of pollen grains from the medium in which they are suspended.

2. The pollen grains are packed tightly together by the proventriculus and are passed as a bolus down to the ventriculus.

3. The boluses pass quite quickly towards the posterior end of the ventriculus (5–20 min.), depending on the concentration and amount of pollen suspension which is fed.

4. The peritrophic membranes do not move down the ventriculus with these packages but pass down at a slower rate.

5. At the posterior end of the ventriculus the pollen may be held up for a considerable time. In the brood-rearing bee it stays there for many hours (up to 12 or more).

In the forager it begins to pass down the hindgut to the rectum after 3 hr. or less.

6. The volume of fluid within the honey stomach, the size of particles in suspension and their concentration have significant effects on the rate and efficiency of filtration by the proventriculus.

7. Some reflexions are made on the possible physiological significance of the mechanism of the proventriculus to the worker honeybee.

I am indebted to Dr C. G. Butler for helpful criticism and for the photographs of my preparations.

I also wish to thank Mr J. K. R. Wood, of the Statistical Department, for his help with the statistical analyses.

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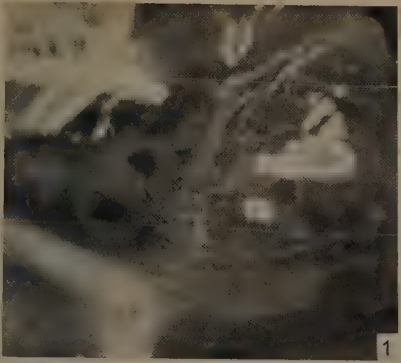
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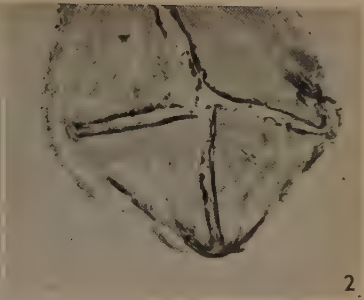
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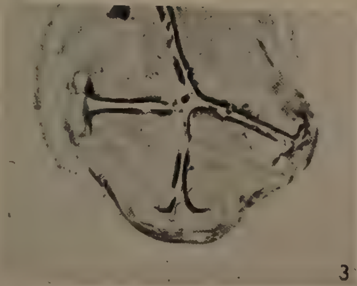
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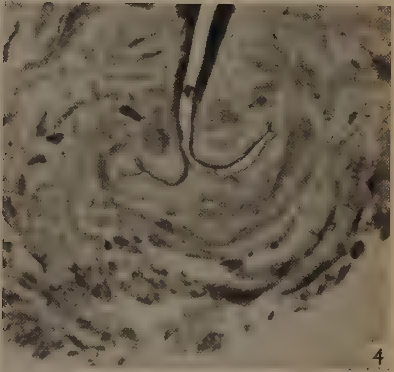
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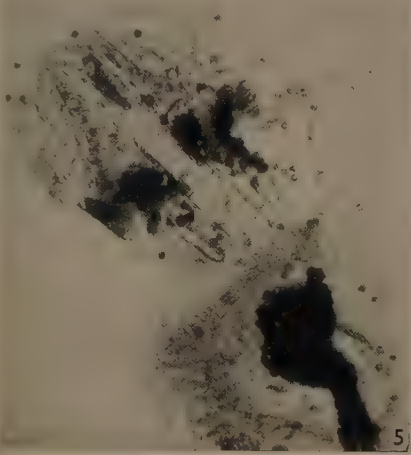
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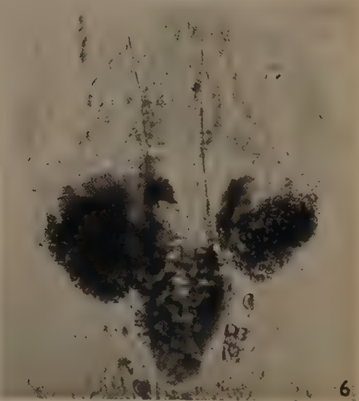
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BAILEY—THE ACTION OF THE PROVENTRICULUS OF THE WORKER HONEYBEE

EXPLANATION OF PLATE 13

Fig. 1. An electronic flash photograph of a window dissection showing two of the four pouches distended with stained pollen. (× 15.)

Fig. 2. Cross-section of the proventriculus where the comb of hairs ceases and before the pouches begin, giving the well known X-shaped lumen. (× 70.)

Fig. 3. Cross-section of the proventriculus showing pouches in the empty, collapsed state. (× 70.)

Fig. 4. Enlarged cross-section of a pouch showing the thick, hyaline, elastic, endocuticular layer with its thin epicuticular layer. (× 308.)

Fig. 5. Whole mount of a proventriculus which was killed as it was passing a bolus back to the ventriculus. (× 70.)

Fig. 6. Whole mount of a proventriculus showing the accumulation of stained pollen within the pouches. (× 70.)

THE RELATIONSHIP OF CARBOHYDRATE METABOLISM AND MITOTIC ACTIVITY IN THE MOUSE

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In recent years much interest has been shown in the biochemistry of mitosis, both as regards the process itself and the agents capable of controlling the frequency of its occurrence in the tissues. This interest has originated in two groups of workers, those biologists who are interested in the fundamental problems of growth and cell division, and the cancer workers who hope, by further understanding these processes, to be able to control the abnormal growth exhibited by tumours.

The regulation of mitotic activity may normally be local (as for example in the healing of wounds), or remote. In the latter case it is probably mediated by endocrine or by the nervous systems, acting either directly, or through an intermediary mechanism. The local regulation must concern both the intrinsic properties of the cells themselves, and also the relations of the various cells in the tissue concerned. The intrinsic mechanisms of mitosis in the cells themselves have so far been studied from the point of view of nucleoprotein synthesis. Following the early work of Kossel, Levene and others (see Jones, 1920), the detailed structure of nucleotides, nucleosides and nucleic acids has been established by the work of Gulland (1947), Plentl & Schonheimer (1944), Muir & Neuberger (1949) and others, particularly since the introduction of methods employing isotopes. From another angle, Caspersson and his school (see Caspersson, 1947) have given some idea of the arrangement of nucleoproteins in the chromosomes, and the time and manner in which this material is increased before mitosis.

The control of the mitotic process through extrinsic agents has until recently been studied from the pharmacological standpoint. Colchicine has been shown to have the property of inhibiting mitosis at metaphase, and latterly other agents which prevent or distort the process have been found, and in some cases used clinically. These include the nitrogen mustards which have been used to treat leukaemia.

Recently the emphasis has shifted more to a consideration of biochemical factors which may play a part in controlling mitosis in the normal animal. In particular, the work of Bullough (Bullough, 1948, 1949, 1950) on the role of carbohydrates in influencing mitotic activity in the epithelium of the mouse ear has suggested a possible mechanism for such control. He has put forward the idea that the frequency of mitosis in epidermal cells is altered by the variations in concentration of sugar in the blood and glycogen in the cells themselves. He has attempted to correlate the diurnal cycle in mitosis known to characterize these cells (see Carleton, 1934; Franklin & Cooper, 1940; Bullough, 1943; and others) with changes in blood sugar and tissue glycogen; further, he claims to have altered the normal cycle by administering carbohydrates parenterally (Bullough, 1949). If his findings were substantiated, an advance would have been made in our knowledge of the biochemical changes in the tissues which affect the frequency of mitosis in such cells, and this in turn might provide a useful weapon for investigators of abnormal mitotic activity.

It was with this latter aspect in view that the present work was begun. It has been shown (Laws, 1951), that in tumour-bearing mice, the frequency of mitosis in the epithelium of the pinna, quite remote from the tumour itself, progressively declines. This fall may be either a toxic effect of the tumour, exerted through the blood stream, or a deprivation effect, in which the tumour depletes the normal tissues of essential nutrients. This latter effect, in turn, might be attributable directly to successful competition by the tumour for materials available in the body in limited amounts only, or indirectly to a depression of the animal's appetite that causes general inanition. With either of these latter possibilities, it seemed that Bullough's lessening of mitotic depression by the administration of carbohydrate might throw some light on whether a lack of this class of substance was in any way responsible for the reduction in mitotic activity observed in the skin of tumour-bearing mice. This has been done with negative results (Laws, 1952), but to control those findings it was thought desirable to repeat on normal animals some of the procedures then followed. The results of these experiments are reported here.

MATERIALS AND METHODS

The mice used were of the Bagg Albino substrain C, bred in the Department through many generations of brother-sister matings; genetically, therefore, they were relatively uniform. They were used when 12–16 weeks old, by which age they are adult and almost fully grown. In view of the great variations in epidermal mitotic activity known to characterize different phases of the oestrous cycle (Bullough, 1943), only male mice were used.

Mitotic counts were made on whole epidermal mounts by the method described in full elsewhere (Laws, 1952). Briefly, this method is as follows. Small pieces of the pinna are removed 5 min. after the application of a depilatory barium sulphide paste, and are transferred to 1 % acetic acid until the surface epithelium can be removed as a single sheet. This is then sandwiched in filter-paper to prevent curling and fixed in glacial acetic acid-alcohol mixture; after hydrolysis in N-HCl, the epithelium is stained in leuco-basic fuchsin according to Feulgen's method. The whole sheet, in which the nuclear material alone is stained, can then be dehydrated and mounted as a permanent preparation. One hundred microscopic fields, each containing about 200 epithelial cells, are selected at random and the mitotic figures counted.

Blood-sugar estimations were made by Haslewood & Strookman's (1939) method, which gives values for the 'true sugar' without the inclusion of other reducing substances. It requires only 0·05 ml. of blood, which is easily obtained from the tail of a mouse; duplicate estimates in a single animal are thus possible. As will be noted below, values for tail blood were closely correlated with those for simultaneous samples of carotid blood.

Kaolin and starch were given as 4 % suspensions, in normal saline, glucose as a 6 % solution.

RESULTS

(1) *Blood-sugar values in carotid artery and tail venous blood*

The following observations were made to determine whether the sugar estimations made with venous blood from the tail could be regarded as representative of the blood level generally. They were made at 10 a.m. and at noon, the times of day at which major differences in the frequency of mitosis in epidermal cells is known to occur. The mice in each group were first bled from the tail and then decapitated; the blood issuing from the severed neck vessels was mainly arterial. The results are given in Table 1. It can be seen that there is a tendency for the carotid arterial blood value to exceed the venous blood value slightly but that the difference is without statistical significance. Similarly, the noon values are both rather lower than the earlier ones, though again the difference is not significant.

Table 1. *Normal blood-sugar values in tail venous and carotid arterial blood in groups of mice sampled at 10 a.m. and noon*

Group	No. of of mice	Time	Blood sugar (mg. %)			
			Tail		Carotid	
			Mean	S.E.	Mean	S.E.
1	19	10 a.m.	118	±4·5	123	±5·0
2	16	Noon	111	±7·2	112	±6·7

(2) *Blood-sugar values in tail venous blood and mitosis counts in epidermal cells at 10 a.m. and noon*

The absence of any significant difference between the blood-sugar values at 10 a.m. and at noon was unexpected, and it was therefore decided to repeat this experiment together with simultaneous determinations of the mitotic activity of the epidermal epithelium. The mice of group 1 were all bled from the tail at 10 a.m. and immediately afterwards a small piece of the pinna was removed; the mice of group 2 were similarly treated at 12 noon. As a further test, a similar experiment was performed with the difference that the same mice were used at both times (group 3). The results are set out in Table 2. It will be seen that there is no significant difference between the mean blood-sugar values for all the groups of mice irrespective of the time of sampling. The changes in the mitosis counts between 10 a.m. and noon, however, are highly significant, and the same alteration is found when the observations are repeated on the same group of animals (group 3) as when they were made separately on two groups (groups 1 and 2). These reductions in the counts at this period of the day are fully in accord with the findings of earlier investigators of the diurnal mitosis rhythm.

Since it seemed possible that the manipulation of the mouse at 10 a.m. might have affected its blood-sugar value at noon, a final experiment was carried out in which both sugar estimations and mitosis counts were obtained at both times but on different days. On the first day, mice 1–4 were bled and had a piece of pinna removed at 10 a.m. and mice 5–8 were treated similarly at noon. The next day, mice 5–8 were dealt with at 10 a.m. and mice 1–4 at noon. The results are shown in Table 3.

Table 2. *Blood-sugar values and epidermal mitosis counts in samples taken at 10 a.m. and noon*

Group	No. of mice	Time	Blood sugar (mg. %)		Mitosis counts (per 100 fields)	
			Mean	S.E.	Mean	S.E.
1	8	10 a.m.	125	±4·3	40	±2·2
2	12	Noon	129	±3·4	12	±1·9
3	28	10 a.m.	124	±4·5	38	±4·0
3	28	Noon	133	±9·0	13	±2·7

Table 3. *Blood-sugar values and epidermal mitosis counts in samples taken at 10 a.m. and noon on different days*

Mouse no.	First day				Second day			
	10 a.m., blood mitosis		Noon, blood mitosis		10 a.m., blood mitosis		Noon, blood mitosis	
	Sugar	Count	Sugar	Count	Sugar	Count	Sugar	Count
1	98	34	—	—	—	—	131	2
2	130	32	—	—	—	—	121	4
3	113	20	—	—	—	—	138	6
4	130	24	—	—	—	—	140	12
5	—	—	99	8	118	32	—	—
6	—	—	105	40	141	32	—	—
7	—	—	98	4	151	26	—	—
8	—	—	110	86	132	52	—	—

It will be found that the mean values for the blood-sugar estimations in this experiment fall within the usual range, with little alteration between 10 a.m. (127 mg. %) and noon (118 mg. %). In some animals there is a fall and in some a rise, when the values for the 2 days are compared. In all the mice in the first group, and in two of the four in the second group, the mitosis counts at 10 a.m. and at noon show the usual difference. Two of the mice in the second group, however, do not show this characteristic decline in the noon count. This finding of a high mitosis count at noon is not exceptional, though from the mean values already recorded it will be clear that it is unusual. The reason for these high figures for mice 6 and 8 are obscure, though they are clearly of such importance as to nullify the factors which normally determine the diurnal epidermal mitosis rhythm.

(3) *The effects of injections of kaolin, starch and glucose on the blood-sugar and epidermal mitosis counts*

As no correlation could be found between the blood-sugar values at different times and the simultaneous mitosis counts, it was decided to examine whether or not the latter might be modified by such procedures as the injection of starch and glucose, both of which might be expected to raise the blood-sugar level. In this experiment each animal was sampled for blood-sugar and mitosis counts at 10 a.m. In the case of starch and kaolin it was then given a subcutaneous injection of 20 mg. in saline and resampled at noon. Control animals received saline. Glucose was given in four doses each of 20 mg. in isotonic solution, at 10 a.m. and 30, 60 and 90 min. later. Control animals received four doses of saline. The results of these injections on the blood-sugar values are shown in Table 4. It can be seen from the table that the injection of starch suspension had no effect in raising the blood sugar appreciably above the level found with a similar injection of saline. At the beginning of the experiment, when, from the previous work of Bullough, it was expected that starch would cause a rise in the blood-sugar level, it seemed necessary to exclude the possibility that any effect produced was due simply to non-specific irritation. A few mice were therefore given a comparable injection of kaolin. The resulting rise in the blood sugar was insignificant. Only when the mice received four injections of glucose during a 2 hr. period did the final blood-sugar level show a perceptible rise. Alone of the four series, did the mice given glucose probably have a circulating blood-sugar level that was above the normal for the greater part of the 2 hr. period. From the mitosis counts,

Table 4. *Effects of starch, kaolin and glucose injections on blood-sugar values 2 hr. later*

Group	No. of mice	Material injected	No. of injections	Time of injection	Blood sugar at noon (mg. %)
1	20	Saline	1	10 a.m.	101 ± 4·0
2	20	Starch	1	10 a.m.	106 ± 5·0
3	6	Kaolin	1	10 a.m.	114 ± 7·5
4	6	Glucose	4	10 to 11·30 a.m.	126 ± 7·0

Table 5. *Effects of starch and glucose injections on the epidermal mitosis counts*

Group	No. of mice	Material injected	No. of injections	Time of injection	Mitosis counts (per 100 fields)	
					10 a.m.	Noon
1	12	Starch	1	10 a.m.	40 ± 5·0	13 ± 0·4
2	6	Glucose	4	10 a.m. to noon	28 ± 4·0	8 ± 1·5

shown in Table 5, it can be seen that the injections of neither starch nor glucose significantly affected the usual phasic decline that occurs between 10 a.m. and noon. Indeed, the resemblance between these findings and the figures recorded in Table 2 for an earlier experiment in which no injections were made is very close.

DISCUSSION

It may be stated at once that the present results do not support Bullough's suggestion that the mitotic activity of epidermal cells in mice is closely correlated with their carbohydrate metabolism. Although the now well-established phasic change in mitosis count in their epidermal cells has been clearly reproduced in the present series of animals, the steep fall in the count between 10 a.m. and noon is evidently unassociated with any comparable change in the blood-sugar level. Nor have attempts to alter the mitotic cycle by the injection of starch or glucose met with any success.

In view of this disagreement, it is desirable to reconsider some of Bullough's findings and explanations. His main observations in normal mice were as follows. First, that the mitotic activity and blood-sugar levels were *inversely* related. Secondly, that on injecting starch (he had little success with glucose), however, there appeared to be a *direct* relationship between the ensuing rise in the blood sugar and the absence of a fall in the mitotic activity at the time expected from a knowledge of the diurnal cycle. Thirdly, that insulin reduced both the blood sugar and the rate of mitosis. And fourthly, in more recent work (Bullough & Eisa, 1950), that the concentration of glycogen in the tissues is correlated with the mitotic activity.

It will be seen that some of these findings are apparently contradictory. He attempts to explain the paradox inherent in the first two observations as follows. He postulates that there is a lack of parallelism between the level of sugar in the blood and that in the cells concerned under different physiological conditions. In normal animals, the blood sugar is highest during the waking hours, but at this time its rapid metabolism lowers its effective level in the cells and consequently their mitotic activity also. During sleep, although the blood sugar is low, cell usage is so reduced that carbohydrates tend to accumulate and mitotic activity is stimulated. The addition of extra carbohydrate in the form of injected starch raises the level of blood sugar sufficiently high, even in active animals, for it to accumulate in the cells and thus stimulate their mitosis. Thus, according to Bullough, it is ultimately the level of cell carbohydrate which determines mitotic activity.

There remain, however, certain of Bullough's experimental observations which are difficult to reconcile with this hypothesis. First is his finding that injections of glucose had no effect upon mitotic activity, although it might reasonably be supposed that such procedures would increase the tissue glycogen irrespective of any marked change in the blood-sugar level. It is difficult to accept his explanation that the differing effects of starch and glucose injections are wholly attributable to their different rates of absorption. Secondly is his observation that the mitosis stimulating effect of starch is reversed if large amounts are injected (Bullough, 1949). For instance, he found that if 50 mg. of starch were given instead of the usual 20 mg., there was no prevention of the usual morning fall in mitosis counts—on the contrary this was accentuated. Thirdly is the effect of insulin. This substance tends to increase the uptake of sugar in the tissues, but, as has been shown by the recent work of Somogyi (1949), the resulting hypoglycaemia produces the release of antagonistic substances which inhibit its utilization. It is possible therefore that this effect is active in Bullough's animals and that the reduction of mitosis found is the resultant of these opposing forces.

While these observations appear to throw some doubt on the validity of Bullough's conclusions, this is not to say that carbohydrate metabolism plays no part in mitosis. On the contrary it is probable that an adequate supply of these substances is necessary for the mitotic process. In the extreme cases of carbohydrate deficiency seen in mice starved or maintained on inadequate diets, this may well be the factor which limits mitotic activity. What is suggested by these results is that in the normal, well-fed animal, which has an adequate supply of carbohydrate, some other mechanism should be sought to explain the diurnal cycle.

SUMMARY

1. The presence of a variation in the mitotic activity of the ear epithelium of normal mice between the hours of 10 a.m. and noon has been demonstrated, in accordance with the 'diurnal cycle' found by other workers. An attempt to correlate this with alteration in the level of reducing sugar in the blood has failed, since this level showed no appreciable variation between 10 a.m. and noon.

2. Efforts to influence either the blood-sugar level or the mitotic activity by injecting starch subcutaneously failed to produce any effect. Injection of glucose produced some slight rise in the blood-sugar level, but did not influence the mitotic activity.

3. It is suggested that, while an adequate supply of carbohydrate is probably necessary for the mitotic process, the diurnal cycle may normally be controlled by nervous or hormonal influences.

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